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Parenteral Nutrition as a Risk Factor for Bronchopulmonary Dysplasia: its Role and Possible Mechanisms in Infants Less than 29 Weeks Gestation

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Cette thèse intitulée:

Parenteral nutrition as a risk factor for bronchopulmonary dysplasia: its role and possible mechanisms in infants less than 29 weeks gestation

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a été évaluée par un jury composé des personnes suivantes :

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Résumé

Une complication dévastatrice de la prématurité est la dysplasie bronchopulmonaire (DBP). Son étiologie est liée au stress oxydatif précoce. L'impact du stress oxydatif non-radical (stress redox) provoqué par les peroxydes contaminant la nutrition parentérale (NP) sur la DBP est peu étudié chez l'humain. Cependant, une réduction de l'incidence de DBP de 25-30% a été atteinte en appliquant une photoprotection complète de la NP (réduisant de moitié la contamination par les peroxydes). Une corrélation entre le potentiel redox sanguin (indicateur du stress redox) au jour 7 de vie et la sévérité de la DBP a aussi été rapportée.

Hypothèse et objectifs:

Hypothèse: chez les grands prématurés, les peroxydes contaminant la NP induisent un stress redox prolongé contribuant au développement de la DBP. Cet effet est causé par une détoxification insuffisante des peroxydes.

Objectifs:

- Vérifier l'impact de la NP sur le stress redox prolongé et la DBP relativement à d'autres oxydants (O_2, infection, transfusion sanguine).
- Examiner différents mécanismes expliquant l'inefficacité de la détoxification des peroxydes.
- Évaluer la mesure des peroxydes urinaires spécifiques comme biomarqueur précoce de DBP.

Patients et méthodes:

Cent-seize nourrissons < 29 semaines de gestation étaient suivis jusqu'à 36 semaines d'âge post-menstruel. Pour le premier objectif, la concentration sanguine de glutathion réduit (GSH) et disulfure (GSSG) a été mesuré à 36 semaines d'âge post-menstruel pour le calcul du

potentiel redox par l'équation de Nernst. Pour le second objectif, le glutathion plasmatique et les activités érythrocytaires de la glutathion peroxydase et réductase (GPx et GR) ont été mesurés à 7 jours de vie. Pour l'objectif 3, l'ascorbylperoxyde (AscOOH) urinaire était mesurés aux jours 3, 5, 7 de vie. Le test χ^2, le test t-Student, l'ANOVA et la régression linéaire (r^2 ajusté) ont été utilisés avec un $P < 0,05$ comme seuil de signification, lorsqu'approprié.

Résultats:

Une NP > 14 jours a induit un potentiel redox plus oxydé (-193±5 versus -203±2 mV) et plus de DBP (89% versus 24%). $FiO_2 \geq 25\%$ au jour 7 était également associé un redox plus oxydé (-191±2 versus -198±2 mV) et plus de DBP (90% contre 45%). La régression logistique suggérait qu'une augmentation de 1% de FiO_2 et une augmentation quotidienne de NP entrainent une augmentation du *odds ratio* pour la DBP de 1,57 (1,09 - 2,28) et 1,17 (1,03 - 1,33). Comparativement aux valeurs normales, la concentration plasmatique de glutathion était faible (1,02, 0,49-1,76 μmol/l: médiane, 25e -75e centile) alors que les activités GPx et GR étaient normales. L'AscOOH urinaire augmentait au fil du temps et était plus élevé chez les nourrissons qui ont développé une DBP.

Conclusion:

La durée de la NP et le supplément d'O_2 ont des effets additifs sur le stress redox prolongé et un risque accru de DBP. Un faible taux de glutathion pourrait limiter la capacité à détoxifier ces peroxydes. Une diminution de la durée ou le développement d'une formulation plus sûre de NP et une thérapie de remplacement du glutathion peuvent être utilisés comme stratégies possibles pour diminuer le risque de DBP chez les grands prématurés.

Mots-clés : Oxygène, nutrition parentérale, glutathion sérique, potentiel redox du glutathion, stress oxydatif, stress redox, ascorbylperoxyde, glutathion peroxydase, dysplasie bronchopulmonaire, nouveau-né prématuré.

Abstract

One of the most devastating prematurity complications is bronchopulmonary dysplasia (BPD). BPD etiology is multifactorial and one major factor is early oxidative stress due to high oxygen exposure. The relationship between non-radical oxidative stress (redox stress) caused by the peroxides contaminating the parenteral nutrition (PN) and BPD is less studied. Studies applying complete photoprotection of PN, which can decrease the peroxide contamination of PN by half, decreased BPD by 25-30%. A correlation between the redox potential on day 7 of life and BPD severity was demonstrated in preterm infants.

Hypothesis and objectives:

Hypothesis: Peroxides contaminating PN lead to prolonged redox stress and contribute to the development of BPD. This effect is due to deficient detoxification of peroxides.

Objectives:

- To study the post PN redox stress duration and its impact on BPD in relation to other oxidants (O_2, infection, blood transfusion)
- To test if the deficient peroxides' detoxification is related to glutathione deficiency
- To evaluate first week urinary ascorbylperoxide concentration as a BPD biomarker

Patients and methods:

One hundred sixteen infants < 29 weeks of gestation were followed until 36 weeks postmenstrual age (PMA). Fifty-one families gave consent for urine and blood samples. The first objective was achieved by measuring total blood reduced (GSH) and disulfide (GSSG)

glutathione at 36 weeks PMA using capillary electrophoresis to calculate the redox potential using Nernst equation. For the second objective, total plasma glutathione, red blood cell glutathione reductase (GR) and peroxidase (GPx) activities were measured on day 7 of life. The third objective was achieved by measuring ascorbylperoxide (AscOOH) in the urine on days 3, 5 and 7 of life. Chi-square, *t*-student, ANOVA, linear regression and repeated measure ANOVA were used as appropriate; $p< 0.05$ was significant.

Results:

PN duration > 14 days was associated with more oxidized redox potential (-193±5 versus -203±2 mV) and more BPD (89% versus 24%). $FiO_2 \geq 25\%$ on day 7 of life was also associated with a more oxidized redox potential (-191±2 versus -198 ±2 mV) and more BPD (90% versus 45%). The effects of PN and FiO_2 on redox potential and BPD were additive. In logistic regression model, each 1% increase in FiO_2 and each day increase on PN resulted in an increase in the OR for BPD by1.57 (1.09 - 2.28) and 1.17 (1.03 - 1.33) respectively. Compared to normal values, total plasma glutathione concentration was low (1.02, 0.49-1.76 µmol/l: median, 25^{th} – 75^{th} percentiles) whereas GPx and GR activities were sufficient. Urinary AscOOH increased overtime (p=0.001) and was higher in infants who later developed BPD or death (p=0.037).

Conclusion:

Early O_2 and peroxides contaminating PN have additive effects associated with prolonged oxidative stress and increased BPD. Extremely preterm infants have low glutathione level that limits their capacity to detoxify peroxides. Higher first week urinary AscOOH levels are associated with increased BPD or death. Judicious use of oxygen decreasing the duration or

developing a safer formulation of PN and GSH replacement therapy should be investigated as strategies to decrease BPD in extremely preterm infants.

Keywords: Oxygen, Parenteral nutrition, serum glutathione, redox potential of glutathione, oxidative stress, redox stress, ascorbylperoxide, glutathione peroxidase, bronchopulmonary dysplasia and preterm infants.

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List of Abbreviations

ANOVA	Analysis of variance
AscOOH	2,3-diketo-4-hydoxyperoxyl-5,6-dihydroxyhexanoic acid (Ascorbylperoxide)
BPD	Bronchopulmonary dysplasia
BW	Birth weight
CIHR	Canadian institutes of health research
CLD	Chronic lung disease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FiO_2	Fraction of inspired oxygen
g	Gram
GA	Gestational age
GPx	Glutathione peroxidase (EC 1.11.1.9)
GR	Glutathione reductase (EC 1.8.1.7)
GSH	Reduced glutathione
GSSG	Disulfide glutathione
H_2O_2	Hydrogen peroxide
kg	Kilogram
MAT	Methionine adenosyl transferase (EC 2.5.1.6)
mg	Milligram

MMPs	Matrix metalloproteinases
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICU	Neonatal intensive care unit
NIH	National institutes of health (United States)
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
O_2	Oxygen
PAS	Pediatric academic societies
PDGF-A	Platelet-derived growth factor A
PMA	Post menstrual age
PMA	Postmenstrual age
PN	Parenteral nutrition
RA	Retinoic acid
RDS	Respiratory distress syndrome
SEM	Standard error of the mean
SNAP	Score for neonatal acute physiology
SOD	Superoxide dismutase (EC 1.15.1.1)
TGF-β	Transforming growth factor-Beta
TIMPs	Tissue inhibitors of matrix metalloproteinases
US	United States of America
VEGF	Vascular endothelial growth factor

I dedicate this work to our brave preterm infants and their families

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Prologue

I was a 10 years old young boy when I had this sever abdominal pain and my father took me to the emergency room. A very calm and smiling physician took care of me. With the gradual relief of my pain I fell in love with this profession. Nothing in is this world equals the satisfaction of changing another person's life. Babies are unique in that they are the most vulnerable patients a physician can care for. This is what attracted to the pediatrics and neonatology. I'm passionate about neonatal care and I enjoy watching every single sick little one gets better.

Get science out of the laboratory

Neonatology is a field in rapid evolution where interventions can have life-long impacts. For example, the surfactant treatment saved so many lives since 1980. At international conferences there are increasing amount of laboratory-based works presented year after year. However, minority of these make their way to clinical research testing and even less are integrated into clinical care. At CHU Sainte-Justine, the laboratory of Dr Jean-Claude Lavoie has produced state-of-the-art research for the last 30 years, with about 70 peer reviewed articles. Unfortunately, only a handful of his work has been translated into human clinical research. the fact that no clinical intervention resulted from this long-lasting amazing work perplexed me greatly. I felt as a clinical researcher that it our duty and mission to bridge the gap between laboratory and clinical research, to ensure that we create real change in preterm infants' lives. With this dream and mission, I started my PhD studies in this laboratory **with a vision of a translational research program**.

When I started this journey, I knew there would be many challenges as laboratory and clinical research are 2 unique fields. I knew there would be intellectual and cultural barriers. Basic science research starts with a hypothesis and designs specific experiments to validate or refute it, with the final aim of gaining new knowledge. Translational research starts with a health need and looks for scientific tools to fulfil that need.

I have savoured every moment of this journey so far. I have put all my efforts in understanding and analysing the works of Dr Lavoie and started actively participating in ongoing laboratory projects. The deep understanding gained helped me conceptualize my translational study and choose the appropriate biological markers that would apply to the clinical setting.

My journey in the laboratory in preparation to this work

I began by reading all the works produced form this lab in the last 30 years. Dr Lavoie's interest in PN began with the discovery of peroxides' contamination of PN (1). His team was first to describe that non-lipid peroxides form more than 80% of peroxides contaminating PN. (2) They were also first in describing the role of light in accelerating peroxide production reaction through riboflavin excitation (3, 4). Afterwards, they documented a reduction in infants' urine peroxides following complete photo-protection of PN (5). This was followed by a randomised controlled trial of photo-protection which demonstrated a 30% reduction of bronchopulmonary dysplasia if the PN was completely photo-protected (6). This study showed many other benefits of photoprotection as well (7, 8). The challenge of integrating photo-protection into daily neonatal practice is the technical difficulty of large-scale complete photo-protection as partial photo-protection was found to be of no benefit (9). The team's research then examined the effect of redox potential changes resulting from PN contamination with

peroxides on lung development (10-13). More recently the team discovered.an important biologically active peroxide that could be specifically measured and named it ascorbylperoxide (13-16). With all this background in mind I started actively participating in ongoing laboratory studies at that time with the team aiming to be able to extrapolate the clinically available biological markers. Being familiar with these biological markers, their limits and their significance was an important step before starting my translational research program in preterm infants.

In the following paragraphs, I will describe the laboratory studies that I participated in. This canvas will outline my own evolution and understanding of the subject matter and highlight the laboratory origin of my translational research program. The first laboratory study was entitled "Ascorbylperoxide from parenteral nutrition induces an increase of redox potential of glutathione and loss of alveoli in newborn guinea pig lungs" and it was published in the Redox Biology journal in May 2014 (17). In this study our neonatal guinea pig model was used. The group receiving an increasing dose of a peroxide contaminating the parenteral nutrition (PN), Ascorbyl peroxide (AscOOH), was compared to a group receiving the same dose of AscOOH with H_2O_2 in a concentration similar to existing PN solution (350 μM). AscOOH alone was associated with dose dependent activation of caspase-3 (the executive enzyme in apoptosis) and decreased alveolar index. The addition of H_2O_2 did not affect the alveolarization index while it decreased the activation of caspase-3. The dose dependent increase of redox potential with AscOOH reached a plateau in the presence of H_2O_2. It is important to note that only H_2O_2 stimulated Nrf2 (transcription factor that controls the expression of antioxidant proteins) and NF-κB (protein complex that controls DNA transcription of many proteins including inflammatory pathway). In this study we showed that some specifically measured PN

peroxides are involved in the induction of hypo-alveolarization (key feature of BPD) whilst also increasing in the redox potential of glutathione. We highlighted the importance of works aiming to develop safer PN compounding or administration strategies. This study was one of the foundations of our second clinical study presented in chapter 3 of this thesis. We suspected that Both H_2O_2 and AscOOH are peroxides that are most likely detoxified by the glutathione antioxidant system involving GSH and GPx. Our laboratory team had already demonstrated that a decrease in guinea pig plasmatic level of GSH was related to inhibition of methionine adenosyl-transferase (MAT) caused by the PN peroxides (18). following this study, the next logical direction was to document weather GSH replacement by adding GSSG to PN could resume the normal pulmonary development.

Consequently, the second laboratory study "Adding glutathione to parenteral nutrition prevents alveolar loss in newborn Guinea pig" was published in the journal of Free Radical Biology and Medicine in October 2015 (19). The gamma-glutamyl transpeptidase enzyme has similar affinity for both GSH and GSSG as a precursor for cysteine that is used for *de novo* production of GSH. In addition, GSSG is much more stable in PN than GSH. These two factors led us to use GSSG addition to PN as a strategy for GSH replacement therapy. In this study we first confirmed that AscOOH is a substrate to GPx in Michaelis-Menten kinetics. Six groups were compared sham, PN with and without photoprotection, 180 µM of AscOOH with and without 10 µM GSSG and PN exposed to light with 10 µM GSSG. The addition of GSSG to PN resulted in decreasing the redox potential and the level of activated caspase-. It also normalized the alveolarization index on histology sections. This work provided a confirmation of our suspected mechanism of PN peroxide contamination on redox potential and alveolar integrity. This paper on GSSG supplement addressed the effect of GSSG supplementation on

the lungs. Our other question was its effect on the liver and specifically on MAT activity as the liver is the most important organ involved in *de novo* GSH production and distribution. This question was addressed in our next laboratory work.

The third laboratory study "Impact of glutathione supplementation of parenteral nutrition on hepatic methionine adenosyl transferase activity" was published in Redox Biology journal in August 2016(20). In this study, we had 6 groups in 2 series of solutions. The first included sham, PN and PN with GSSG. The second included dextrose infusion, dextrose with H_2O_2 and dextrose with AscOOH. The MAT inhibition was more pronounced in the groups with PN as its activity was decreased by 45 ± 4 % compared to 23±7% decrease in the peroxides' groups without amino acid and lipid supplement. The hepatic MAT activity correlated significantly with the redox potential, but the magnitude of the effect was different according to amino acid and lipid supplement (peroxides solutions versus PN). In addition, the use of dithiothreitol (DTT) reversed the effect of peroxides solution without methionine supplement but the inhibition of MAT persisted in the PN group. This indicated that the inhibition cannot solely be explained by a peroxide-oxidation of this thiol function and that other molecules must be involved. This study emphasized that with the current approach of PN compounding and administration, prevention of peroxide formation or GSH replacement to correct the redox potential is not sufficient in this model to restore the MAT activity.

With my initial reading and understanding of Dr Lavoie's contributions and the added personal growth by participating in the previously described research studies I believed that translational research to test these results in human preterm infants is the logical next step before proposing the solutions created in Dr Lavoie's laboratory to help prevent BPD.

As such, this thesis aims to test the hypothesis that there is an association between the PN contaminated with peroxide and the outcome of BPD through redox potential changes. In addition this thesis will test if the mechanism suggested by our guinea pig model, a decreased GSH limiting peroxides detoxification, is pertinent in human preterm infants.

Living in many worlds!

For the last few years, I have truly appreciated the diverse nature of being a clinician researcher with translational research interest. Specifically, while fully immersed in my basic science projects and the translational work under Dr Lavoie's direction, I also designed and conducted separate clinical research projects These clinical projects were focused on osteopenia of prematurity, early neonatal hyperbilirubinemia and PN related liver disease including the role of ursodiol in its treatment (21-24). From the 8 articles I generated during my PhD period, I have chosen to present in this thesis two articles that were directly in line with the translational objective under the supervision of Dr Lavoie. I'm proud of this accomplishment and feel that this life changing experience will help propel me as a distinguished translational research scientist in the filed of oxidative stress in neonatal medicine.

Chapter 1: Introduction

1.1 Prematurity

1.1.1 Incidence, definition and classification

Each year, around 15 million babies are born preterm around the world (25). Prematurity is defined as live births with a gestational age (GA) less than 37 weeks. GA refers to the number of completed weeks after the onset of the last menstrual period. Prematurity is a worldwide major health problem ranging between 5% to 18% of all births across different countries with very serious health and economic consequences. In Canada, during the year 2013 there were 380,323 births, of which 29,716 (7.8%) were preterm births (26). While all infants less than 37 weeks gestational age are all considered preterm, all prematurity related complications are increasing with decreasing gestational age at birth. That is why this World Health Organization categorization of preterm infants based on their gestational age is important when comparing different outcomes in different preterm infants' populations:

- Extremely preterm infants < 28 weeks GA
- Very preterm infants ≥ 28 to < 32 weeks GA
- Moderate to late preterm ≥ 32 to < 37 GA

1.1.2 Causes of preterm birth

Preterm birth is an outcome that could be initiated by multiple mechanisms. The most known mechanisms include infections, inflammation, placental insufficiency or hemorrhage and uterine anomalies or over distension (27, 28) yet spontaneous preterm birth can occur without any identifiable mechanisms of these mentioned above. In addition to spontaneous

preterm births the category of medically indicated preterm delivery is well recognized and saw an increase in its percentage of all preterm births in the last few years (estimated at around one third of all preterm births) (28). This category includes maternally indicated deliveries as severe preeclampsia or fetus indicated deliveries as severe intrauterine growth restriction (29). The fact that we fail to predict and/or prevent preterm birth despite several years of research and numerous clinical studies reflects the complexity and variability of causes (30).

1.1.3 The Costs of prematurity

In a relatively recent and one of the most comprehensive evaluations of preterm birth costs, Institute of Medicine (US) Committee on Understanding Premature Birth and Assuring Healthy Outcomes estimated the societal cost of prematurity in the USA annually to be up to $26 billion (31). In addition to the maternal delivery and early neonatal intervention costs this evaluation included disability-specific lifetime medical, special education, and lost productivity costs for four specific developmental disabilities that are associated with preterm birth (cerebral palsy, mental retardation, vision impairment, and hearing loss). On individual level the cost was inversely correlated to gestational age with infants less than 28 weeks GA at $ 190,467 on the year of birth, infants between 28- and 31-weeks GA at $ 94,785, and between 32 and 36 weeks GA at 13,621 compared to $ 3.325 for the full term newborn (31). It should be noted that one of the limitations of this analysis is that it did not include lifetime costs of the caregiver due to lack of adequate data. These out-of-pocket costs incurred by the families of preterm infants include the cost of transportation, accommodations, and childcare for other siblings during hospitalizations as well as during outpatient visits (29, 32). The heavy

cost of prematurity on societal and on individual level makes it a priority to support the research aiming to prevent preterm birth or to prevent any of its major complications.

1.1.4 Overtime evolution of survival and outcome of preterm infants

Due to the better understanding of the pathophysiology, in addition to the technical and pharmaceutical advancements the rate of preterm infants' survival has increased dramatically in the last few decades with a marked decrease in the mortality rate even for the smallest infants. While a preterm 1 kg infant chance of survival was about 5% in 1960 it reached 95% in 2000 (33). A large recent cohort including 355 806 infants born between 2000 and 2009 with birth weight of 501g to 1500 g, mortality rate decreased from 14.3% to 12.4% (34). This improvement of survival was accompanied by an improvement in outcomes. While outcome of infants between 1000 to 1500 g was poor in the 60s now most of them are doing well (35). However, with the increase of survival at lower gestational age the number of infants with disabilities has stayed approximately the same (33).

1.1.5 Short- and long-term complications of prematurity

Prematurity is now recognized as the leading cause of mortality among children under five years of age (25). In addition, preterm infants are at risk of having many perinatal complications including respiratory distress syndrome, nosocomial infections, necrotizing enterocolitis, intraventricular hemorrhage and retinopathy of prematurity (35, 36). This initial respiratory distress syndrome could precede the development of bronchopulmonary dysplasia (BPD), the most frequent and most severe complication in extremely preterm infants (37-39). It is important to note that the prematurity related complications are not limited to the perinatal period but could also extend far beyond this period. Many respiratory, metabolic and

neurocognitive complication are now recognized to be caused by preterm birth (37, 40-44). The origin of these multiple short- and long-term complications is related to the abrupt change in environment from the intrauterine to extrauterine while being in a period of active organogenesis and organ maturation. This change in environment negatively affects the normal development of fetal organs particularly the lung which is the primary subject of this thesis.

1.2 Normal lung development and prematurity

The main function of the respiratory system is to provide the organism with external oxygen and remove excess carbon dioxide from the blood and this function takes place in the lungs. However, the respiratory system includes many organs that are necessary to its function. It is composed of the upper and the lower respiratory tract. The upper respiratory tract filters, warms and moistens the air and it includes of the nasal cavities, sinuses, nasopharynx and the larynx above the vocal cords. The lower respiratory system is composed of the conducting system and the lung. The conducting system includes the larynx below the vocal cords, the trachea, the main bronchi, and the bronchioles that distribute the air throughout the lung. The lung itself is composed of respiratory bronchioles, alveolar ducts and the alveoli, the place of oxygen and carbon dioxide exchange. The pulmonary blood vessels are part of the lung. The pulmonary arteries bring the deoxygenated blood with excess carbon dioxide from the heart to the lung and the pulmonary veins return oxygenated blood with normal carbon dioxide to the heart. In this section, I will describe the normal development of the lung with special emphasis on lung alveolarization and the impact of antenatal conditions as well as preterm birth on this process.

1.2.1 Normal lung development

Five overlapping chronologic stages characterize the human lung development (Figure 1). They describe the structural and histological changes that occur during the lung morphogenesis and maturation (Figure 2). These stages include the embryonic, pseudoglandular, canalicular, saccular and alveolar stages extending throughout the gestation period into the postnatal period.

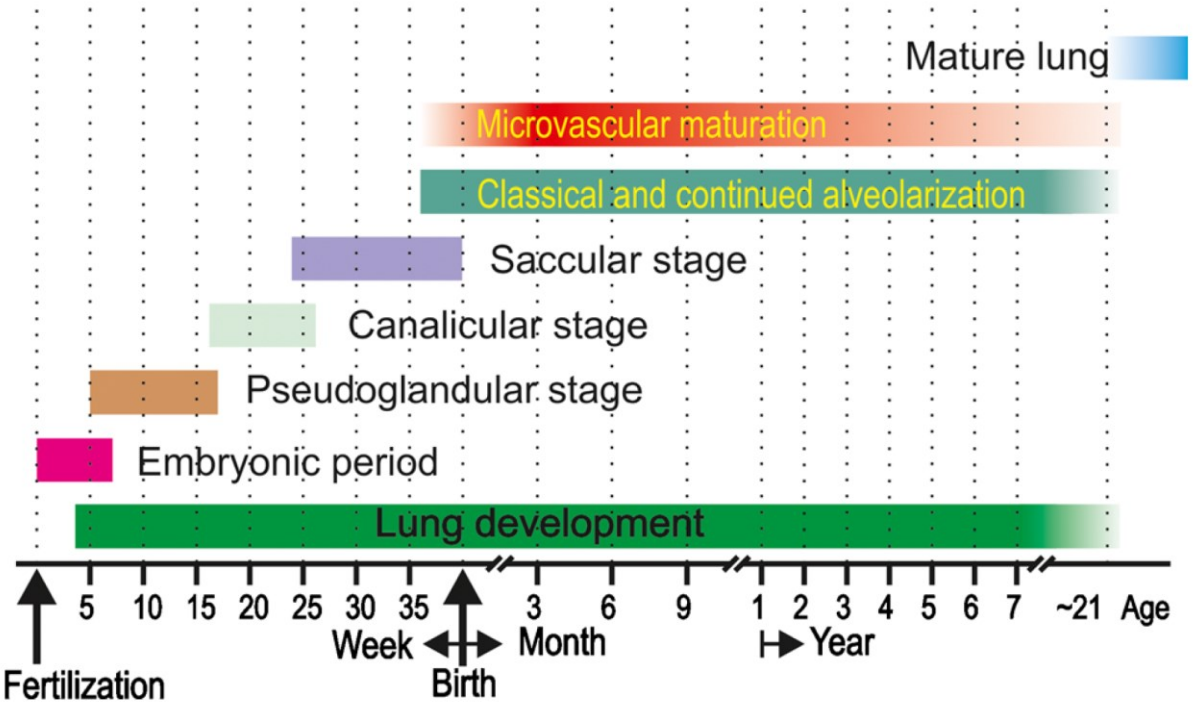

Figure 1. Human lung development time scale.

As the processes of lung development are starting centrally and spread peripherally, the stages of lung development are overlapping. From (45) under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/)

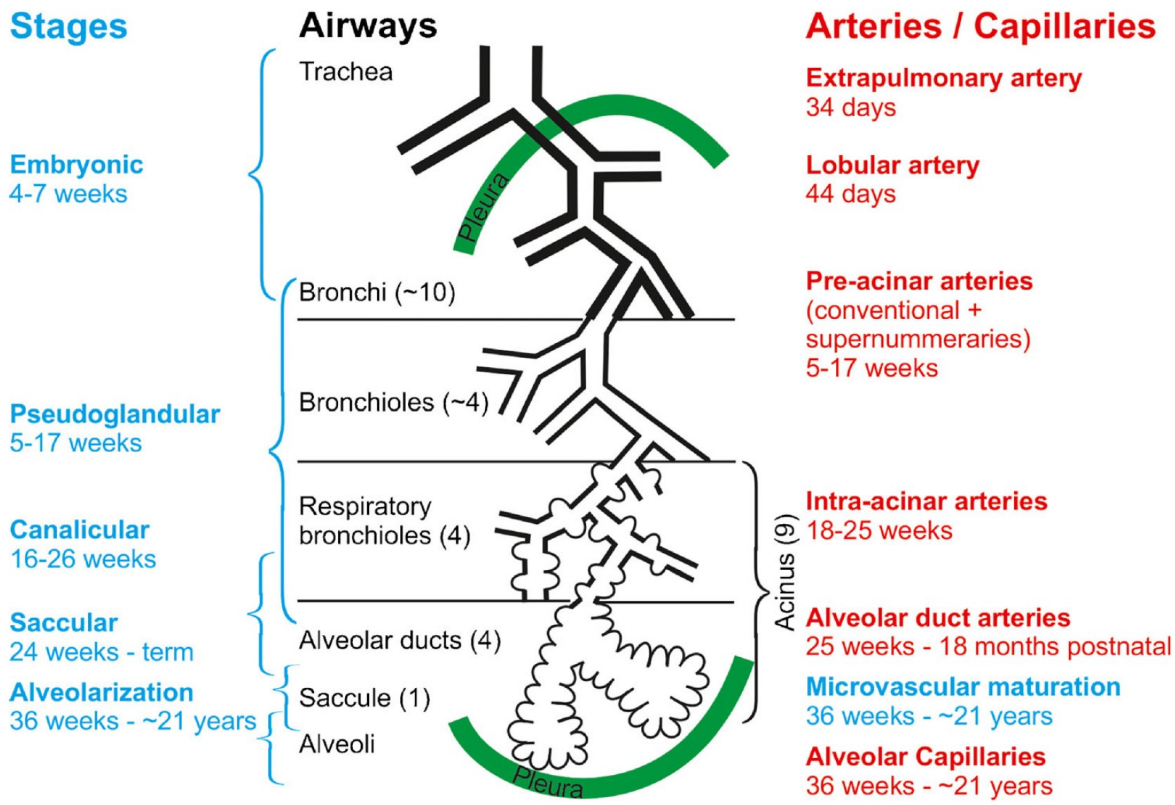

Figure 2. The lung development stages.

The stages of lung development (blue) are correlated to the development of the airways (black) and the arteries (red). From (45) under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/)

1.2.1.1 Embryonic stage (3-7 weeks GA)

During this phase lung bud arises from the ventral foregut endoderm. From the trachea primary, secondary, tertiary bronchi arise by branching morphogenesis. Pulmonary arteries develop from the aortic arches and pulmonary veins develop as outgrowth of the left atrium. Autonomic innervation extends to the trachea and bronchi during this period (45, 46).

1.2.1.2 Pseudoglandular stage (5-17 weeks GA)

Branching morphogenesis continues during this period and the tracheal tree formation is completed by 17 weeks of gestation. In the tracheal, cartilage and mucus glands develop and serous, ciliated, mucus, basal neuroendocrine cells differentiate during this period. In distal lung, respiratory bronchioles, acinar buds/tubules form in this time frame. The pulmonary vascular and lymphatic systems continue their development; pre-acinar blood vessels form the distal mesenchyme. Parallel to airway branching pulmonary arterial development continues. Pulmonary lymphatics arise from veins. Pulmonary lymphatics and veins extend into interlobular septa. The autonomic innervation also parallels airway branching all through this period (45, 46).

1.2.1.3 Canalicular stage (16-26 weeks GA)

While the mesenchyme thins, canalicular and acinar tubules lengthen, subdivide and widen. This period is characterized by the formation of the primitive future alveolar capillary network with the beginning of the formation of the blood air barrier. At the cellular level, type I and type II alveolar cells differentiate. Surfactant starts to be synthesized and stored in the lamellar bodies of type II cells (45, 46). This stage witnesses an increase of fetal lung fluid production and the initiation of the fetal breathing like movements.

1.2.1.4 Saccular stage (24-38 weeks GA)

Fluid filled saccules as acinar buds expand. Saccular distal airspaces continue to grow and branch. Alveolar septal walls arise as mesenchymal condensation. These septa contain well formed double capillary network. Alveolar septal crest formation is marked by elastin deposition at its sites. At the cellular level, type I alveolar cells flatten and elongate. Type II

cells gradually produce more surfactant. With these changes in lung structure, air breathing and gas exchange become feasible. However, premature infants born during the saccular stage of lung development are at high risk to complications related to biochemical immaturity of the lung (lung surfactant deficiency) leading to the respiratory distress syndrome or hyaline membrane disease. This acute lung injury during the saccular stage can alter the subsequent alveolar growth and differentiation leading to BPD (45, 46).

1.2.1.5 Alveolar stage (36 weeks GA to young adulthood)

This stage is characterized by a dramatic increase in the gas exchange surface area caused by the subdivision of the primitive saccular wall (primary septa) by new inter air-space walls (secondary alveolar septa) resulting in new alveoli. These secondary alveolar septa partition the transitory ducts and terminal saccules into true alveolar ducts and alveoli. The number of alveoli in human lung increases very rapidly during the first 2 years of life and recent human studies demonstrated an increase with slower rate throughout adolescence (45, 47). At the cellular level, there is marked interstitial fibroblast proliferation and differentiation with increased collagen, elastin and fibronectin deposition. Both type I and type II alveolar cells rapidly increase in number, but type II cells are the ones proliferating actively suggesting that type II cells are progenitor cells for type I cells. While type II alveolar cells represent two thirds of all alveolar epithelial cells in human adult lung, it only represents about 7% of total alveolar surface while the larger squamous type I cells account for the rest of the surface area. Surfactant production by type II alveolar cells significantly increases in this period. Although type I alveolar cells form a tight barrier that is impermeable to ions and fluid, they are easily injured by oxidants, infection and barotrauma (46). This stage is also characterized by the

maturation of the alveolar-capillary membrane. Throughout this stage the secondary alveolar septa lengthen and thin and its interstitial tissue is reduced. Another main characteristic is the remodelling of the capillary bed is the fusion of the two septal capillary networks, a capillary network on each side of a central core of connective tissue, into one. Pulmonary vascular resistance decreases because of this pulmonary vasculature and capillary bed remodelling. Injury to the lung early during this stage of development may result in abnormal lung remodelling with a reduction of number of alveoli. The most illustrative pathology of impaired alveolar multiplication in human is BPD, the most common chronic respiratory complication of prematurity. Impaired alveolarization (alveolar hypoplasia) and altered microvascular maturation are the main pathophysiologic features of BPD. In the next section, I will discuss the phases of alveolarization process and the mechanisms regulating it.

1.2.2 Alveolarization phases and its regulation

The time line of this process is relatively long; starting around 36 weeks of gestation and continuing till early adulthood (45). While definitive alveoli can be found in the lungs as early as 36 weeks of gestation, the average number of alveoli in a full-term baby is about 150 million (110 to 175 million). This very rapid increase in alveolar number (also called bulk alveolarization phase) continues during the first 6 months of life when 430 million (250 to 710 million) alveoli are already formed. The alveolarization rate then decreases progressively until about 8 years of age when lung growth becomes proportional to body growth. By adulthood the alveolar number reaches an average of 480 million (275 to 790 million) (47-49).

Each of the following factors is playing a central role in the regulation of alveolarization:

1.2.2.1 Vascular endothelial growth factor (VEGF)

The expression of VEGF and its receptor peaks in the developing lung during the bulk alveolarization (50). Several experiments in the developing rat involving VEGF receptor inhibitors demonstrated that normal angiogenesis is required for normal alveolarization (51, 52). In these experiments rarified peripheral vessels and decreased airspace-parenchyma ratio were noted. These observations were confirmed in extremely preterm baboon, with impaired VEGF found in animals developing BPD (53). Treatment with Recombinant human VEGF treatment enhanced alveolarization in hyperoxic lung injury model of neonatal rats (54, 55).

1.2.2.2 Elastogenesis

The generation of elastic fibers following the incorporation of elastin into microfibril bundles is called elastogenesis. Experimental approaches that disrupt this process elucidated the essential role of elastin in distal lung development. Fewer, dilated distal air sacs with attenuated septa were found upon inactivation of elastin gene in mice (56). Animals in this model die shortly after birth before reaching the alveolarization phase. Another model used Platelet-derived growth factor A (PDGF-A), chemoattractant of myofibroblasts, to demonstrate the role of elastin deposition in alveolarization indirectly (57, 58). Platelet-derived growth factor A- deficient mice surviving postnatally develop lung emphysema with failure of alveolar septation. In this model the absence of secondary septation results from a profound reduction of elastin deposition. As both fibroblast growth factor (FGF) and retinoic acid (RA) are key regulators of elastogenesis their role in lung alveolarization will be discussed in the next section.

1.2.2.3 Fibroblast growth factor (FGF)

FGF increases the expression of lysyl oxidase and tropoelastin, both are essential for elastogenesis, in myofibroblasts (59). FGF is also involved in the signaling for migration of alveolar myofibroblasts during postnatal alveolarization (60). FGF receptors (FGFR) 1 to 4 are present in the developing lung with increased expression of FGFR 3 and 4 during alveolar formation (61).

Homozygous disruption of FGFR 3 and 4 genes in murine model resulted in animals with normal lungs at birth but with completely blocked alveologenesis. These animals were not able to form secondary septa to produce new alveoli (62). This failure in secondary septation production was not noticed with either single mutant. Neonatal rats exposed to hyperoxia had reduced expression of both FGFR 3 and 4 (63).

1.2.2.4 Retinoic acid (RA)

RA enhances the gene expression of tropoelastin (64) and PDGF-A (65). In vitamin A mild deficiency neonatal rats, the number of alveoli was reduced as well as the total alveolar surface area (66). Treatment with retinoic acid resulted in 50% increase in alveolar number in neonatal rats (67). In rat hyperoxia model, RA treatment from day 3 to day 14 resulted in normalisation of the alveolarization with no significant difference from control group on day 42 of life, while hypo-alveolarization persisted in hyperoxia-exposed group that was not treated with RA (68). These observations were confirmed in premature infants as well. Preterm infants < 32 weeks GA who developed BPD were found to have significantly lower concentration of plasma vitamin A compared to the group without BPD (69). In a recent

Cochrane review of 9 studies, vitamin A supplement appeared to be beneficial in decreasing the risk of oxygen needs or death at one month of age in infants < 32 weeks GA (70).

1.2.2.5 Matrix metalloproteinases

One of the very important elements of normal lung development is extracellular matrix remodelling. It is important to realise that almost 40% of synthetized collagen will be degraded in few hours during bulk alveolarization in neonatal rat and this will allow the interstitium to become thinner and less cellular (71). Matrix metalloproteinases (MMPs) and namely MMP 2 and MMP 14 are actively involved in this remodelling and their expression and activity increase progressively during rat lung alveolarization (71, 72). Tissue inhibitors of MMPs (TIMPs) modulate the MMPs' activity to create the required balance (73). The importance of MMPs in lung alveolarization was highlighted by the finding that low MMP 2 concentrations in tracheal aspirate of preterm infants was significantly and independently associated with the development of BPD (74).

1.2.2.6 Transforming growth factor-Beta (TGF-β)

TGF-β is a 25 KD protein implicated in cellular proliferation and differentiation (75). The TGF-β and its signaling were found to be essential for normal late lung development (76). The important role of TGF-β in lung alveolarization was documented as conditional overexpression of TGF-β resulted in a phenotype similar to histologic picture of BPD in neonatal mouse lung (77). In neonatal C57BL/6J mice hyperoxia model, exposed to 85% FiO_2, TGF-β signaling was potentiated with increased susceptibility of alveolar type II cells to TGF-β induced apoptosis (78). Moreover, in the murine hyperoxia model, treatments with TGF-β-neutralizing antibodies improve pulmonary alveologenesis and vasculogenesis (79). In human

preterm infants < 30 weeks GA, abnormally high concentration of TGF-β was found in the endotracheal secretions of infants who later on developed sever BPD with need to home O_2 therapy (80).

1.2.2.7 Hormonal regulators

Steroids have significant short and long-term effects on the lung development as demonstrated in cases of antenatal and post-natal administration for fetus and preterm infants. Antenatal administration of corticosteroids for women at risk of preterm birth is a standard of care that is supported by the most recent Cochrane review including 30 RCT with 7774 women and 8158 infants (81). In this meta-analysis antenatal steroids were shown to decrease neonatal mortality and RDS after preterm delivery. In sheep model, antenatal corticosteroids were associated with a decrease in the lung mesenchyme, an increase in the lung airspace and mRNA for surfactant proteins within 24hours of administration (82, 83). It should be noted that in the same model, decreased alveolar septation was noticed 7 days after steroids administration (82). When some treated fetuses were allowed to continue till term without further treatment, the neonates had decreased lung capacity compared to untreated fetuses. Thus, the beneficial effect of antenatal steroids seems to be limited to its short-term action (84). In post-natal life, the main effects of postnatal dexamethasone include the acceleration of alveolar wall thinning and the two capillary layers fusion (85-87). While these acute effects ameliorate the respiratory condition, this process prevents further septation and results in fewer and larger alveoli (85-87) which was found to be persistent in adult rats (86). It is also to be noted that postnatal dexamethasone does not affect endothelial cells replication, but it decreases the replication of fibroblasts and type II cells.

The thyroid hormone plays an important role in lung maturation and alveolarization (88, 89). Compared to control mice and euthyroid offspring of hypothyroid mother, postnatal hypothyroid mice offspring of hypothyroid mothers had decreased postnatal alveolarization due to decreased alveolar septation resulting in fewer large saclike alveoli (90). More recently, a model of iodine deficiency in female Sprague Dawley rats' pups were compared to iodine sufficient mothers matched age pups. Structural wise, larger and irregularly shaped alveoli were documented in the iodine deficient pups (91). This resulted in reduce tidal volume, peak inspiratory and expiratory flow, and dynamic lung compliance in iodine deficient pups compared with iodine sufficient pups when double-chambered plethysmograph assessment was performed (91). In addition to these structural changes, significantly lower concentrations of surfactant protein B and C were observed in iodine deficient pups indicating significant delay of lung maturation (91).

It should be noted that all these mechanisms controlling lung development and maturation are programmed for the normal intrauterine environment. Preterm birth with the significant transition to extra-uterine life before the appropriate lung development and maturation by term gestation leads to significant consequences that will be addressed in the next section.

1.2.3 Prematurity effects on lung development

In utero the placental unit takes care of all gas exchange needed by the foetus. Programmed developmental changes in lung morphology and physiology occur in preparation to delivery near term so that this transition from a relatively hypoxic environment to ambient air environment causes minimal impact. Preterm birth disrupts this preparation and leads to birth with immature lung that is not ready for ambient air breathing. This lack of preparation

leads to specific pathologies in the acute phase 'respiratory distress syndrome' and over longer period of time 'chronic lung disease of prematurity (CLD) or bronchopulmonary dysplasia (BPD)'. In this section we will discuss the normal lung preparation before term birth first and then we will address both the acute phase pulmonary pathological changes related to preterm birth.

1.2.3.1 Pulmonary preparation for term delivery

The goal of the morphological maturation that is described in the **1.2.1** section is to increase the surface area for gas exchange and to decrease to minimum the thickness of the alveolar-capillary membrane. This goal is met by secondary septation leading to the creation of alveoli and the fusion of the 2 capillary beds in the secondary septa into one single capillary bed, in concomitance with thinning of the matrix of alveolar septa (45, 92, 93). Infants born 24 weeks of gestation are born during the transition from canalicular to saccular stage of lung development which is much less effective in gas exchange compared to near term infants at 36 weeks of gestation who started the formation of lung alveoli (45). This explains the need for mechanical ventilation assistance and the need of O_2 supplement in the extremely preterm infants group.

Another important factor that helps near term neonatal smooth transition is the adequate production of surfactant. Surfactant is essential in reducing surface tension at air-liquid interface in the airspaces (94, 95). While surfactant components can be detected within type II alveolar cells as early as 20 weeks GA (96), evidence of its presence in the amniotic fluid is not present until 26 weeks GA (96, 97). In addition, detection of surfactant proteins B and C occurs later in development around 30 weeks GA (during the saccular stage) (96). Many of the

preterm infants who are born before late third trimester are at high risk of developing hyaline membrane disease or respiratory distress syndrome due to surfactant deficiency that leads to alveolar collapse and increased work of breathing (96).

One other very important developmental preparation before near-term birth is the switch of secretory activities from chloride secreting membrane that produce the lung fluid necessary for normal lung growth to sodium absorbing membrane. Without this switch the presence of fluid in the potential airspace and the interstitium can impede gas exchange as diffusion is much faster in gas phase than in water (98, 99).

Another cornerstone aspect of developmental preparation for near term birth is the rapid upregulation of antioxidant defenses near the end of gestation. This part will be discussed in more details in **section 1.4**.

Preterm birth means simply that these essential developmentally programmed changes will not or will partially take place. This will result in difficult transition from the intrauterine to ambient air environment with variable degrees of respiratory failure that is known in the acute phase as respiratory distress syndrome (30).

1.2.3.2 Respiratory distress syndrome (RDS)

RDS is also known as hyaline membrane disease and is by far the most common cause of respiratory distress in preterm infants. Its name highlights the importance of surfactant deficiency in the pathogenesis of the disease (30, 100). Alveoli with insufficient surfactant tend to collapse. This alveolar collapse increases the necessary work of breathing to re-open the alveoli during inspiration leading to respiratory distress. This repetitive opening and collapse of alveoli leads to shear stress that damages the fragile lung architecture with leakage

of proteinaceous debris into alveoli forming the 'hyaline membrane' (30, 101). Clinically the preterm infants have tachypnea, chest wall retraction, grunting and in severe cases cyanosis. A 'ground glass' appearance on the chest X-ray represents the diffuse atelectasis and the 'air bronchogram' reflects the contrast between the airless parenchyma and the air-filled bronchi.

In near term infants, surfactant replacement can lead to rapid improvement with independent spontaneous breathing with no marked long-term consequences. While the surfactant deficiency is a major contributor to the pathology of RDS, treatment with exogenous surfactant will not be enough to achieve independent spontaneous ventilation in the most extremely preterm infants due to the structural immaturity described in **1.2.3.1**. In these infants needing prolonged neonatal respiratory support, the acute pulmonary pathology progress toward a chronic lung disease that is known as bronchopulmonary dysplasia (30).

1.3 **Bronchopulmonary dysplasia (BPD)**

Since the term of BPD was coined 50 years ago by Northway et al, it has become both the most common serious complication of prematurity and the most common form of chronic lung disease during infancy (102-105). Concomitant to the increasing rates of survival of extremely preterm infants, the incidence of BPD continues to be high especially in infants less than 28 weeks GA (106-108). Despite the enormous research and clinical advancement efforts for several years the impact on the incidence, severity and long-term outcomes of BPD is relatively minor (106). In this section I will discuss the diagnostic criteria, the epidemiology, the short- and long-term consequences and including the related costs as well as the pathogenesis of BPD.

1.3.1 The diagnostic criteria of BDP

One of the particularities of BPD is that it is defined by its treatment and not based its pathophysiology. This led to frequent changes in nomenclature and may be decreased our ability to understand and follow the progression of this important pathology (109, 110).

In the original paper that described BPD, the disease was characterized by prolonged cyanosis, O_2 requirements clinically and by radiologic changes resulting from a chronic lung disease that represents prolonged healing of RDS under the effect of O_2 toxicity and mechanical ventilation (105). The definition of BPD diagnosis was largely debated and concluded in the U.S. National Institutes of Health (NIH) workshop held in 1979 that proposed the BPD definition of "continued O_2 dependency during the first 28 days plus compatible clinical and radiographic changes" (111). With the increase of very preterm infant survival, this definition became less relevant and in 1988 a large cohort study concluded that the need of O_2 at 36 weeks postmenstrual age better predicted abnormal pulmonary findings at 2 y of age than the oxygen therapy requirement at 28 d of life (112). This definition was followed by a severity dependent definition that was proposed by a NIH workshop (113). In this definition the need of O_2 for 28 days of life indicated mild BPD whereas if this need continues (> 21% FiO_2 but < 30%) till 36 weeks postmenstrual age in infants < 32 weeks GA (or 56 days of life in infants > 32 weeks GA), this defines the moderate BPD and if FiO_2 needs are ≥ 30% this is categorized as severe BPD (113). This definition was validated using data from 4688 infants in a retrospective study that concluded that this definition accurately predicts pulmonary outcomes including percent of patients needing treatment with pulmonary medications and rehospitalization for pulmonary causes by 18-22 months corrected age (114).

It is obvious that all the previous definitions have an inherent limitation, which is that the need for oxygen is determined by physicians or nursing staff rather than by a physiologic assessment with predetermined saturation targets. This led to the development of the physiologic definition of BPD that requires an oxygen room air challenge for all infants who need FiO_2 < 30% and classify infants as having BPD if only they fail to maintain their Saturation > 90% for 30 minutes in room air following O_2 weaning at 36 weeks postmenstrual age (115). A follow up validating study showed that many of the infants judged by the treating team as needing O_2 at 36 weeks postmenstrual age did maintain their saturation > 90% during the room air challenge (116). In this study while 35% of infants were classified as having BPD with O_2 needs at 36 weeks postmenstrual age, only 25% had BPD according to the physiological definition.

This wide variability in the definition of diagnostic criteria of BPD stimulated the recent NIH study group, Prematurity Respiratory Outcomes Program, to compare these definitions in a prospective multicenter study that concluded that the evolution in the management of preterm infants (like the wide use of high-flow nasal cannula) limits application of existing definitions and can potentially lead to misclassification. The study pointed out the need for a contemporary definition of BPD that correlates with respiratory morbidity in childhood (117).

1.3.2 Epidemiology of BPD

In the recent cohort of infants between 22- and 28-weeks GA that is including 8515 infants between 2003 and 2007, Stoll et al. reported the incidence of BPD depending on the definition used. While BPD severity definition resulted in an incidence of 68% of all infants

(27% mild, 23% moderate and 18% severe BPD), this incidence was down to 42% using the definition of O_2 need at 36 weeks postmenstrual age and to 40% if the physiologic definition was used (103). The same previously mentioned group of Stoll et al. extended their time interval between 1993 and 2012 and included all infants between 22 and 28 weeks GA in the biggest cohort of 34 636 infants and reported an increase in the BPD incidence defined as O_2 needs at 36 weeks postmenstrual age from 32% in 1993 to 45% in 2000, then a decrease to 40% in 2008 close to their previous report (103, 118).

Other studies reported different incidences of BPD depending on the population used. In a recent cohort of very low birth weight infants (infants less than 1500g), the Vermont Oxford group reported rates between 26.2% and 34.2% between 2000 and 2009 with significant decrease of BPD overtime (34). A comparative study between Canada and Japan neonatal research networks between 2006 and 2008, infants less than 1500 g, the incidence of BPD defined as the need of O_2 at 36 weeks postmenstrual age was 12.3% and 14.6%respectively (119). It should be noted that the saturation target differed between institutions in these studies (34, 103, 119). In a very recent study (2011-2013) that compared the current BPD definitions and their limitations in 765 infants between $\geq$ 23 weeks GA and < 29 weeks GA, the incidence of BP was found to be 40.8, 58.6, and 32.0% of infants, respectively, with O_2 needs at 36 weeks postmenstrual age, severity and physiologic definitions respectively (117).

This incidence makes BPD the most common serious complication of premature infants with 12 000 to 14 000 new cases diagnosed each year in USA alone (104, 120).

1.3.3 Short and long-term consequences of BPD

BPD carries heavy short- and long-term consequences. In the neonatal initial hospitalization BPD is associated with increased risk of impaired postnatal growth, higher mortality and prolonged hospitalization (121, 122). After discharge, infants with BPD are at higher risk of rehospitalisation -especially in the first 2 years of life- and to increased postnatal mortality (123-125).

Long-term consequences of BPD include pulmonary complications including asthma (126). Adults with BPD history are twice like to report wheezing and three times likely to use asthma medications (127). Studies using CT imaging in adults with history of BPD reported emphysema to be common in this population (128). Pulmonary hypertension is another major long-term complication of BPD (129).

In addition to long-term pulmonary complications many children with the history of BPD will suffer from cognitive impairment and neurodevelopmental deficit (130-132).

1.3.4 Economic impact of BPD

While there are relatively many studies reporting the economic impact of prematurity, fewer studies discussed the economic short and long-term impact of BPD. This could be related to the fact that it is difficult to dissect the cost of BPD from the total cost of co-existing prematurity complications. In the elegant work by Johnson et al. that included 425 surviving VLBW infants between 2005 and 2009, the estimated direct cost of the initial hospital stay for infants without BPD was 44,465 ± 23,300 US $ compared to 103,151 ± 43842 US $ for infants who have BPD (133). When trying to dissect direct costs related to BPD, it came to

31,565 US $ (133). It should be noted that these direct hospital costs do not account for physician fees or for family's direct and indirect expenses. Recently, the article from Alvarez-Fuente group in Spain went a little bit further trying to estimate the economic impact of BPD in premature infants who do not have any other prematurity complications in the first two years of life. While these costs where between 45,050 € and 118,760 €, it increased for those who needed O_2 treatment at home to 48,032 – 121,742 € compared to a cost of 910 € for term infants with no complication (134).

With all the above described heavy short- and long-term consequences and costs, BPD became a research priority with expected health, social and economic high impact of any intervention that can lead to a significant reduction in its incidence or severity.

1.3.5 Pathogenesis of BPD

About 50 years ago when BPD was first described, it was a result of the exposure of moderate to late preterm infants to high oxygen and invasive ventilation support. This 'old BPD' was characterized by severe airway epithelial lesions (hyperplasia and metaplasia), airway smooth muscle hypertrophy, extensive diffuse fibrosis, and decreased internal surface area and alveoli (135, 136) . With the advances in neonatal care, specifically: the increasing use of antenatal steroids, the administration of surfactant, the use of less invasive ventilation and improved neonatal nutrition, the number of surviving very and extremely preterm infants increased with a remarkable shift of the pathologic picture of the disease. Now, the new BPD is characterized by decreased, large and simplified alveoli, decreased and dysmorphic capillaries, and negligible fibrosis and airway epithelial lesions (135, 136) . It appears that these lesions of arrested alveolar and vascular development are mainly related to the fact that

most of these infants are born at the late canalicular and early saccular phase with exposure to certain factors that leads to arrested lung development.

While it is widely accepted that the pathogenesis of BPD is multifactorial, there are many controversies about the role of each specific pathogenic factor. In the following paragraphs I will critically summarize the most accepted contributing factors.

1.3.5.1 Oxidative stress and BPD

Historically oxidative stress related to long duration of high O_2 exposure was considered the principal cause of BPD (105, 137). In addition, in the rodent animal models, the exposure to high O_2 without mechanical ventilation is enough to produce structural lung changes similar to BPD (138). These historical cohorts and animal models depend on creating hyperoxia and increased oxidative stress. This situation is much less relevant to the current clinical practices where the minimal necessary supplement of O_2 is provided under continuous saturation monitoring. It was not till late nineties when a large animal model was used in a situation that mimics clinical settings (extreme prematurity, antenatal steroids, postnatal surfactant, intravenous parenteral nutrition, gentle ventilation and minimal O_2 supplements guided by oximetry). This study permitted to conclude that while mechanical ventilation and O_2 toxicity despite being still important factors in the development of BPD, their role is likely not central in the new BPD, as observed in current practice (139). This conclusion became more accepted following the evidence provided by multiple large multicenter studies using two different oxygen saturation targets and comparing their effects on neonatal mortality and morbidities (140-143). The meta-analyses of these trials suggested no effect on the incidence of BPD (144, 145). Putting these data together suggest that while hyperoxia and oxidative

stress were major historical contributors to BPD etiology, the present clinical practices of minimal O_2 supplement and avoiding hyperoxia made the interventions targeting further decrease in O_2 supplement less relevant and may be harmful. On the other hand, more recently, studies on the oxidative stress related to parenteral nutrition contamination with peroxide emerged in early nineties and continued to prove relation to BPD development in both in animal and human studies (1, 4, 6, 10, 12, 146, 147). This aspect will be discussed in more details in the section 1.4.

1.3.5.2 The role of inflammation

Another well-known factor associated with BPD development is inflammation. Substantial evidence indicating that inflammation plays an important role in the pathogenesis of BPD and it was summarized in multiple reviews (148-150). While antenatal inflammation was initially considered a risk factor for BPD development (151, 152), recent large well-designed studies question this relation (153). This controversy could be explained by the variation in the fetal inflammatory response, fetal gestational age, the severity and the duration as well as the causative organism. Some animal studies even reported accelerated lung maturation following induction of lung inflammation (154). However, postnatal sepsis was consistently shown to increase BPD risk (155, 156).

1.3.5.3 The vascular hypothesis

VEGF plays a major role in the pulmonary vascular development that is very closely related to alveolarization. The 'vascular hypothesis' of BPD emphasizes the importance of alteration of VEGF signaling in the pathogenesis of the disease. In animal model, oxidative stress -induced by hyperoxia- decreased VEGF expression (157). When using specific VEGF

receptor inhibitor both vascular growth and alveolarization were decreased (52, 158). Data from human preterm infants found that the concentrations of VEGF in the tracheal aspiration and urine were decreased in infants who will subsequently develop BPD (159-161) .

1.3.5.4 Progenitor cells and BPD

In neonatal mice model of hyperoxia induced BPD, oxidative stress induced by hyperoxia markedly reduced bone marrow, circulating, and lung endothelial progenitor cells (162). This observation was supported by the finding that the presence of decreased endothelial progenitor cells in the cord blood of the extremely preterm infants was associated with higher risk of developing BPD (163). It should be noted that endothelial colony-forming cells in preterm infants were found to be more susceptible oxidative stress induced par hyperoxia (164). In animal model, administration of endotracheal mesenchymal stem cells prevented arrested alveolarization in vascular growth in chronic hyperoxia induced BPD in neonatal rats (165). In addition, similar protector effect was found upon administration of mesenchymal stem cell conditioned medium indicating that may be this protector effect is mediated by stem cells secreted products (166).

1.3.5.5 Genetic polymorphisms and BPD

Many studies tested the role of genetic polymorphisms in BPD pathogenesis. Genetic polymorphisms related to the previously discussed important pathogenic factors were found to be associated with BPD. For example, polymorphism of inflammatory cytokines and Toll-like receptor were strongly associated with BPD (167-171). Other studies suggested an association between VEGF genetic polymorphism and BPD (172, 173). It is of note that the polymorphisms in oxidative stress and antioxidants were of the most frequently found

association with BPD (174-177). These studies were discussed in the recent elegant review article by Dani et al. (178).

1.3.5.6 Common final pathway to BPD development

While the pathogenesis of BPD is usually described as multifactorial, many elucidate to a common final pathway. In addition, the interrelation between these important factors is evident. If we take the example of oxidative stress (in the mice hyperoxia model), it was associated with a significant decrease in VEGF expression (157), and a marked increase in TGF-β expression (its role in alveolarization was discussed in the section 1.2.2.6) (179) and the concentration of proinflammatory cytokines (180). These facts lead to the strong widespread belief that oxidative stress resulting from the imbalance between oxidant load and antioxidant capacity probably plays an important role in the development of BPD (181, 182).

1.4 Oxidant load and antioxidant defenses in preterm infants

Figure 3 depicts a general view of oxidant – antioxidant interaction. The main components of both systems and their interrelation will be described in the following section.

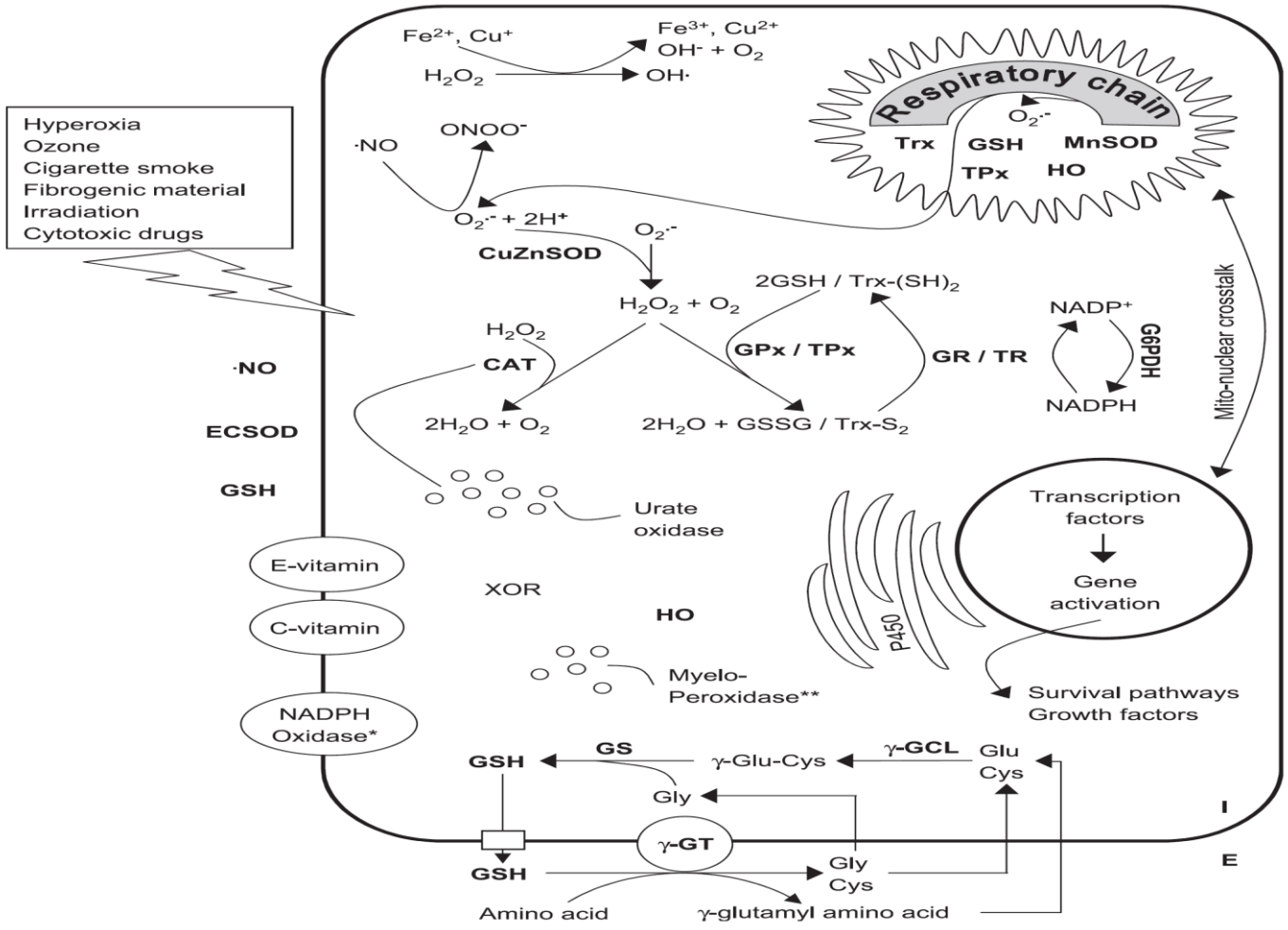

Figure 3. Significant intra- and extracellular sources of oxidants and antioxidants.

The most important intra (I) and extra (E) cellular oxidants and antioxidants are shown in this figure with CAT, catalase; CuZnSOD, copper–zinc superoxide dismutase; Cys, cysteine; ECSOD, extracellular superoxide dismutase; γ-GCL, gamma glutamate-cysteine ligase; Gly, glycine; G6PDH, glucose 6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GS, glutathione synthetase; GSH, reduced glutathione; GSSG, oxidized glutathione; γ-GT, gamma glutamyl transpeptidase; H_2O_2, hydrogen peroxide; HO-1, hemeoxygenase 1; MnSOD, manganese superoxide dismutase; NADP, nicotinamide adenine

dinucleotide phosphate; $^{\bullet}NO$, nitricoxide; $O_2^{\bullet-}$; $OH^{\bullet}$, hydroxyl radical; $ONOO^-$, peroxynitrite; TPx, thioredoxin peroxidase; TR, thioredoxin reductase; Trx, thioredoxin; Trx-S_2, oxidized thioredoxin; Trx-$(SH)_2$, reduced thioredoxin; XOR, xanthine oxidoreductase; *, in phagocytic cells; **, in neutrophils. From (183) with reuse license # 4335690239398.

Preterm birth implies the foetal transition from a relatively hypoxic intrauterine environment to a relatively hyperoxic environment without passing by the well described developmentally programmed preparation which includes a significant increase in antioxidant capacity before term birth. This means that the preterm infants with a precarious antioxidant defense is obliged to face an increased oxidant load which can leads to oxidant-antioxidant imbalance usually called oxidative stress. In the following section I will discuss oxidant sources and types in general, the preterm infants' exposure to many oxidants as part of their regular neonatal intensive care treatment or complications as well as the antioxidant system and the particularities of preterm infants' antioxidant defenses.

1.4.1 Oxidants in the context of prematurity

1.4.1.1 Oxidants: sources and types

Oxidants are produced under normal circumstances as part of oxidative phosphorylation in the mitochondria. Through various protein complexes in the mitochondria O_2 is easily reduced to H_2O_2. A small percentage of O_2 (approximately 1%) will be partially reduced and will result in the production of reactive O_2 intermediate including the superoxide anion radical, hydrogen peroxide and the hydroxyl radical in the presence of trace amounts of metals such as iron or copper, corresponding to the reduction steps by one, two and three electrons respectively (184, 185). Ions and molecules formed because of incomplete reduction

of O_2 are commonly called reactive oxygen species (ROS). These ROS can be further classified into free radical oxidants which include the superoxide anion radical, hydroxyl radical and nitric oxide and the nonradical oxidants which include hydrogen peroxide, hydroperoxy fatty acids, aldehydes, quinines as well as peroxynitrites (186). These oxidants can also be formed during detoxification of xenobiotics, however it should be noticed that a main producer remains the oxidative phosphorylation in mitochondrion (187). While low rate production of oxidants can be considered as normal product of aerobic metabolism, their production rate can be significantly elevated under some pathophysiological conditions like x-irradiation, hyperoxia, shear stress, inflammation and ingestion of some dietary compounds including quinones (184, 188).

1.4.1.2 Different oxidants to which preterm infants are frequently exposed

Inspired oxygen is one of the most frequent oxidants to which preterm infants are exposed. Preterm infants with minimal respiratory distress and who needs only positive pressure support with room air (21% oxygen) with arterial partial pressure of O_2 about 75 mmHg are still considered exposed to supra-physiologic O_2 compared to their counterpart fetuses with arterial partial pressure of O_2 ranging around 25 mmHg (189). Furthermore, most extremely preterm infants are having respiratory distress syndrome (discussed in 1.2.3.2) and they are even given supplemental O_2. It is known that dissolved oxygen available for oxidative phosphorylation in the mitochondria is proportional to oxygen load concentration. Accordingly, there is a marked increase in rate of ROS with increasing O_2 tension, under conditions of hyperoxia. For example, under FiO_2 of 95% there is a 10 folds increase of ROS comparing to ambient O_2 (21%) (190).

Other strong oxidants frequently administered inadvertently to preterm infants, are the peroxides contaminating the parenteral nutrition (PN) (1, 2, 16). Parenteral nutrition is essential to extremely preterm infants' survival as it permits to provide them with proteins, lipids, glucose, minerals and vitamins while they cannot fulfil their nutritional needs due to their gastrointestinal immaturity. Infants less than 29 weeks GA need parenteral nutrition for days up to weeks before they can be on full enteral feeds (191). PN contains strong oxidants as dissolved O_2 and its interaction with reducers such as polyunsaturated fatty acids, amino acids and vitamin C generates oxidant molecules including aldehydes and hydroperoxides derived from lipid peroxidation and hydrogen peroxide (H_2O_2) and ascorbylperoxide derived from ascorbic acid (1-3). These reactions are further induced by photoexcitation of riboflavin (4, 16).

Other situations in which preterm infants are exposed to more oxidants on a less systematic basis include mechanical ventilation (shear stress), blood transfusion, neonatal infections, and necrotizing enterocolitis (188, 192-198)

1.4.2 Prematurity and antioxidant defenses

From the previous section, it is obvious that preterm infants are more exposed to higher oxidant load either as part of birth transition, necessary treatments or complications. It is now important to discuss their antioxidant defenses. I will start with a general discussion of antioxidants and then discuss the developmental regulation of the antioxidants defenses with emphasis on the effect of prematurity. As antioxidant defenses against peroxides and glutathione are in the heart of this thesis' studies; the last two points of discussion in this section will be devoted to these items.

1.4.2.1 Antioxidants' definition and types

An antioxidant was initially defined as 'any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate' (199). This definition evolved overtime and now includes wider range of antioxidant activity that includes molecules existing in relatively high concentration like albumin and the repair of any damage caused by oxidants, so the revised definition most widely used now is 'any substance that delays, prevents or removes oxidative damage to a target molecule' (200). This wide definition includes compound of both non-enzymatic and enzymatic nature (Figure 4).

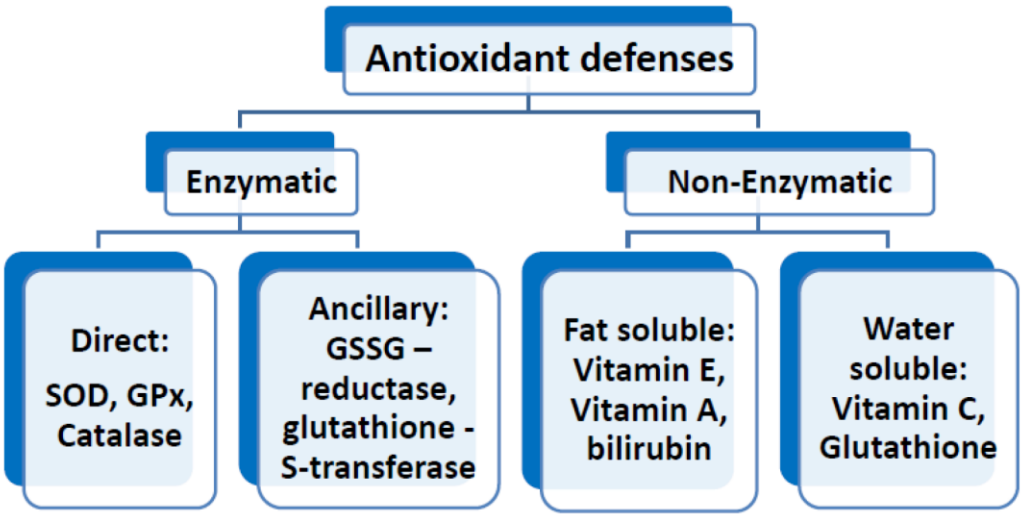

Figure 4. Summary of important antioxidants.

It goes without saying that the best way of antioxidant defense is to completely avoid the oxidant, which is clearly impossible in the case of preterm infants.

It is important to realize that there is no best antioxidant. In fact, the antioxidants' diversity is the best way to match the diversity of oxidants. One of the classic examples of how this diversity responds to a real need is the O_2 partially reduced to the radical oxidant molecule superoxide anion ($O_2^{\cdot -}$) in the mitochondrion. Two superoxide anions are then converted by superoxide dismutase enzyme (SOD) into the non-radical oxidant hydrogen peroxide (H_2O_2) and oxygen (201, 202). In addition to its presence in the mitochondria (Mn-SOD), SOD is also present in the cytoplasm (CuZn-SOD) (203). It is important to recognize that H_2O_2 is a two electron, non-radical oxidant, which is produced at 1 to 4% of the rate O_2 consumption and therefore represents a major oxidant. The complete detoxification of H_2O_2 into H_2O and O_2 is possible by two enzymes glutathione peroxidase (GPx) and Catalase (CAT) (204, 205). When H_2O_2 is not detoxified, it can react through Fenton reaction with Fe^{2+} producing the hydroxyl radical ($\cdot OH$) which is highly reactive. $\cdot OH$ can oxidize proteins, lipids and DNAs.

Another large category of antioxidants is non-enzymatic antioxidants (Figure 4). It includes fat soluble substances (in cell membrane for example) and water-soluble substances (mostly in the plasma and in the cytoplasm). Fat soluble non-enzymatic antioxidants include vitamin E and β carotene while water soluble non-enzymatic antioxidants include vitamin C and glutathione (GSH). Vitamin E embedded in the cell membrane scavenges free radicals and protect it against oxidative damage while being oxidized to inactive vitamin E (206). Vitamin C is a strong antioxidant that participates in a variety of oxido-reductive reactions and allows the recycling of the oxidized vitamin E into its active form (207). The now oxidized inactive form of vitamin C can be reduced to its active form by action of GSH system (208). This diversity and complementarity between antioxidants are the best strategy to face the diversity of oxidants to which the living organism is exposed.

1.4.2.2 Developmental regulation of antioxidant defenses

Many developmentally programmed preparations take place near term. The most classic and well-known example is the increase of lung surfactant synthesis and secretion to prevent hyaline membrane disease (discussed in the section 1.2.3.2). One can speculate that the dramatic change from the in utero hypoxic to the O_2 rich ex-utero environment should require specific preparations. In the following paragraphs we will discuss the evidence of such programmed preparation both in different species of animal model and in human subject and including enzymatic as well as non-enzymatic antioxidants.

In animal model, it was as early as 1976 when lung antioxidant enzymes (specifically SOD) was reported to be lower in fetus compared to adult animal in both rats and rabbits (209). The observation that in late gestation there is a rapid increase of antioxidant enzymes SOD, CAT, GPx came 2 years later when in a rate model a significant increase in these enzymes was observed between 2 days before term and at birth (210). From the same group Frank et al designed a rabbit model study where the timed-gestations were interrupted at 2 1, 22, 26, 28, and 30 d (full term 31.5 days) and postnatally the pups were used at <1-h old, 3-4-d-old, and 5-6-d-old to measure the concentration of lung antioxidant enzymes. This study confirmed a sharp increase in the concentration of these enzymes (110% to 200) in the last 10 to 15% period of gestation (211) (Figure 5). This group extended their study to four different species (rat, rabbit, hamster, and guinea pig) and measuring the same 3 lung antioxidant enzymes. Their studies revealed that essentially similar pattern of antioxidant enzyme maturation exists in the 4 species (212).

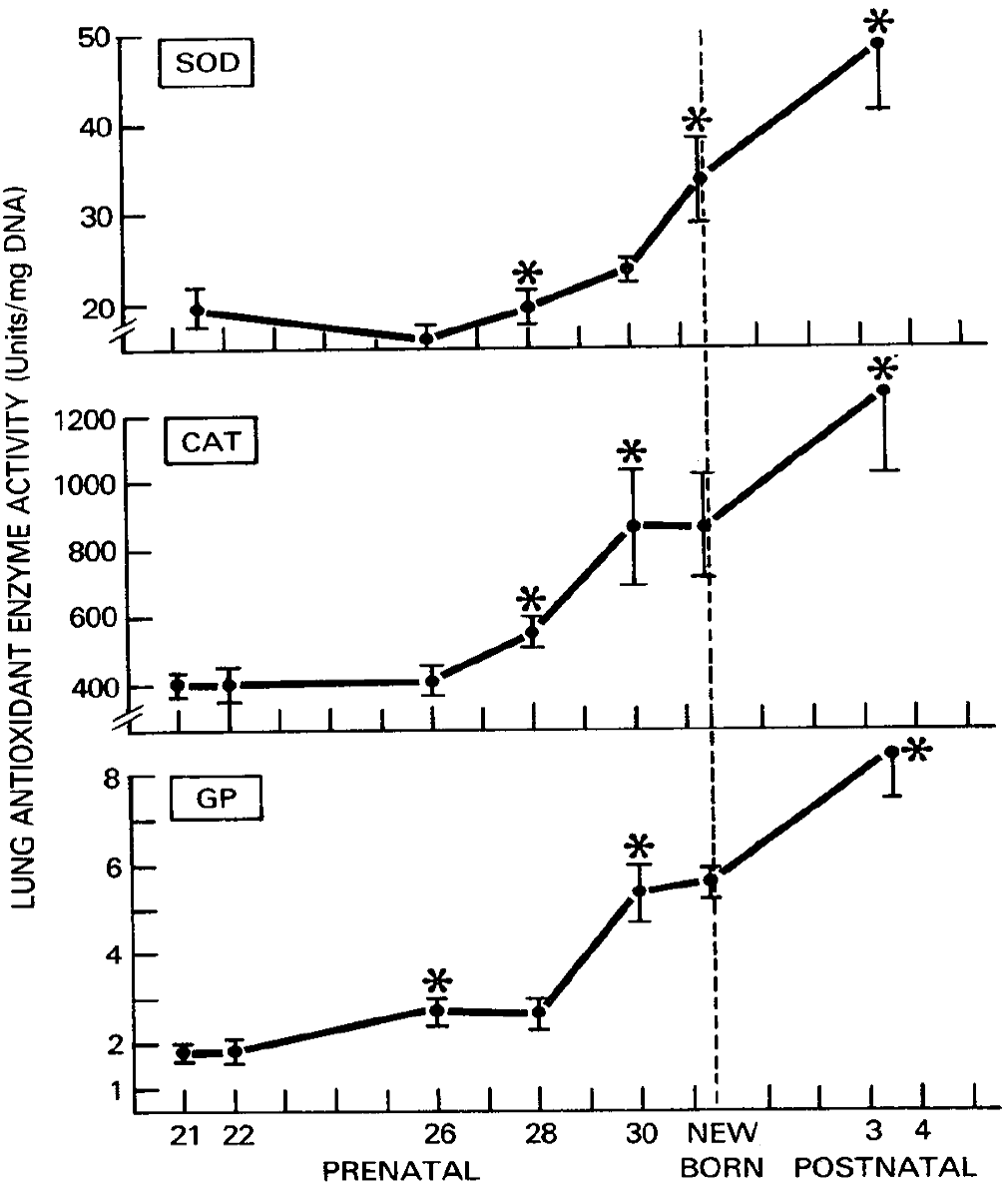

Figure 5. Developmental changes in antioxidant enzyme activity in lungs of fetal and newborn rabbits.

Antioxidant enzyme activity (U/mg DNA) in lungs of fetal and neonatal rabbits. Values are mean±1 SE and *P < 0.05 versus preceding age point value. From (211) licence for reuse # 4335460690654.

In human studies, results of the few small studies are inconsistent. One of the earliest studies evaluating developmental changes in SOD had relatively small number of participants (209). In this study fetal lung tissue was obtained after abortion (18-20 weeks GA). Postmortem adult lung tissue was obtained from patients aged 43-70 years who died from non-pulmonary causes. The Superoxide dismutase activity in human lung tissue increased from 109±11 units/g lung in the fetal tissue (n=6) to 152±32 units/g lung in normal infants 18 hours to 2 months of life (n=3), while it was 182±19 units/g lung in adults (n=5). This increase in activity in normal infants compared to fetus did not reach statistical significance due to small sample size. In another study with relatively bigger sample size, McElroy put in evidence a developmental increase in CAT activity with an increased from 20.9 +/- 7.8 U/mg protein (n = 29) at 11-20 weeks GA to 73 +/- 27.5 U/mg protein (n = 30) following normal delivery (41-60 weeks post-conceptual age) with P less than 0.001 (213). In this study the SOD and GPx activities remained stable. Few years later, Asikainen et al examined the evolution of mRNA and activity of SOD, CAT and GPx in fetuses (n=6, delay for sample collection 1hour), newborns' samples were obtained from 3 autopsies (n=3) with a delay less than 12 hours while adults' samples (n=7) where from patients undergoing lung surgery with macroscopically normal lungs with a delay of less than 1 hour. It should be noted that some of the adults were smokers and others were not. In this study the lung mRNA expression of SOD, and CAT increased, whereas GPx was unchanged toward adulthood. Pulmonary activities of SOD were unchanged, whereas CAT increased 3-fold from fetuses to adults (214). It should be noted that all these studies were limited by ethical challenges of tissue collection leading to small sample size, heterogeneous population and significant delay before tissue collection and

hence despite being suggestive of gradual increase of antioxidant enzymes they cannot be conclusive.

While the antioxidant enzymes seem deficient in preterm infants other studies suggest non-enzymatic antioxidants to be defective in preterm infants as well. Several studies put in evidence the inadequate maternal-fetal transfer of vitamin A, E, C and their low level in preterm infants (215, 216). This was also noted for GSH concentration in the plasma as well as in the tracheal secretions. The GSH level seemed to positively correlate with the gestational age (217, 218). The next point will discuss the antioxidant defenses against peroxides in general and then we will devote the last point of discussion in this section to glutathione, a cornerstone of the glutathione peroxidase family.

1.4.2.3 Antioxidant defenses against peroxides

Cells have multiple defenses against peroxides. These include catalase, peroxiredoxins and glutathione peroxidases.

Catalase is confined to peroxisomes in most cells (with the exception of RBC and neutrophils) (219, 220). The primary function of catalase is to remove H_2O_2 generated by peroxisomal oxidases. Catalase breaks down 2 molecules of H_2O_2 to water and O_2. Mitochondria and the endoplasmic reticulum contain very little, if any, catalase. Any *in vivo* production of H_2O_2 in these organelles can not be disposed by catalase, except if it diffuses to the peroxisomes (219).

The family of peroxiredoxins enzymes reduces H_2O_2 and organic peroxides (221). Peroxiredoxins are ubiquitous and abundant, comprising up to 0.8% of total soluble protein in mammalian cells (222). In peroxiredoxins, a “peroxidatic” cysteine residue serve as the oxidation site by peroxides to cysteine sulfenic acid, which then react with another cysteine

residue to form a disulfide. This disulfide is then reduced by thioredoxin which in turn is regenerated by thioredoxin reductase and NADPH (219, 223). At least 3 classes of peroxiredoxins are known: the most common one is the typical 2-cys peroxiredoxins (forming interchain disulfide), the atypical 2-cys peroxiredoxins (forming intramolecular disulfide) and the 1-cys peroxiredoxin. In the last class, GSH regenerates the -SH group catalyzed by glutathione-S-transferase enzyme.

The glutathione peroxidase (GPx) family was first discovered in animal tissue in 1957 (224). GPx remove H_2O_2 by reducing it to H_2O_2 coupled with oxidation of GSH to GSSG. GPx can act on H_2O_2 and other peroxides like fatty acid hydroperoxides (221). It is present in animal cell cytosol, mitochondria, nucleus, peroxisomes. It is also found extracellular fluids like the lung lining fluid, seminal fluid, milk and amniotic fluid. In our neonatal animal PN model both H_2O_2 and AscOOH were both detoxified by the action of GPx (19). As the glutathione is essential for this reaction; more information about GPx is included in the next section that is dedicated for glutathione.

1.4.2.4 Glutathione: a major antioxidant and determinant of redox status

The GSH metabolism includes its synthesis and degradation. The tripeptide γ-glutamyl-cysteinylglycine or glutathione (GSH) is the most abundant low molecular-weight peptide present in cells (225). Due to it is ubiquitous presence in all cell types at millimolar concentration (0.5-10 mM), it is considered the major non-enzymatic regulator of intracellular redox homeostasis. Glutathione synthesis passes through two ATP-dependent sequential reactions catalyzed by glutamate-cysteine ligase (GCL) -formerly known as γ-glutamylcysteine synthetase- which is the rate-limiting enzyme, and glutathione synthetase

(GS) (226). The two main control points of GSH production is the availability of cysteine and the GCL activity which is feedback inhibited by GSH (225, 227). GSH synthesis is most active in the liver and then with exported via plasma and bile to other organs (225). The whole body GSH reserve is mostly intracellular with about 85% of GSH in the cytoplasm, 10-15% in the mitochondria, and a small percentage in the endoplasmic reticulum (228). In comparison to its intracellular millimolar concentration, the concentration of GSH is far less in the plasma where it is micromolar while it is about 140 times the plasma concentration in the pulmonary lining fluid - where gas exchange occurs- of the same person (229).

GSH is a high turnover molecule with estimated half-life of 2-3 hours in rate liver. The unusual γ-carboxyl peptide bond linking glutamate and cysteine (instead of the usual α-carboxyl peptide bond) is subject to hydrolysis with only one known enzyme γ-glutamyltranspeptidase (GGT). This enzyme is only present on the external surfaces of cells which makes GSH resistant to intracellular degradation (225, 230) . GGT is considered a cornerstone in GSH homeostasis as it provides a continuous source of cysteine through the γ-glutamyl cycle (Figure 6).

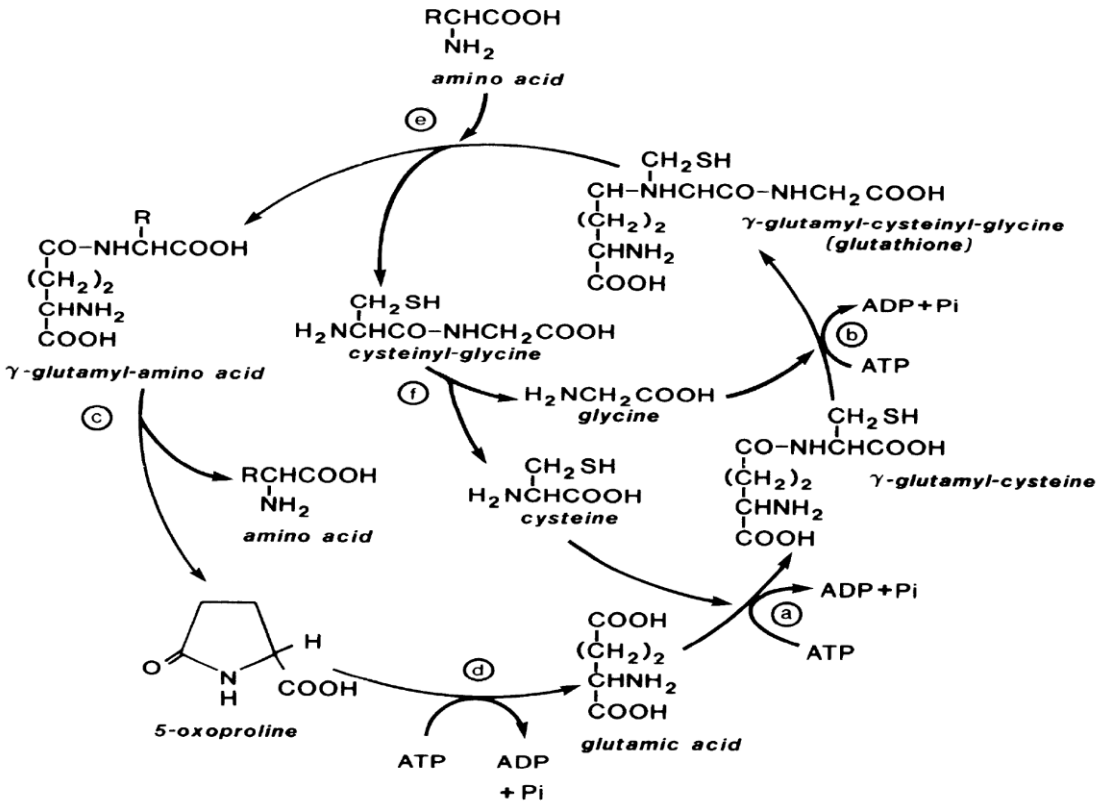

Figure 6. Glutathione metabolism.

Glutathione synthesis and degradation where a is γ-glutamyl-cysteine synthetase, b is glutathione synthetase, c is γ-glutamyl cyclo-transferase, d is oxo-prolinase, e is γ-glutamyl-transpeptidase and f is peptidase (230). Reuse permission is not required.

GSH has many important functions. In the following section I will summarize the most important ones:

One of the most important functions is its antioxidant capacity. As described in 1.4.2.1 GPx can detoxify H_2O_2 and lipid peroxides to water and corresponding alcohol respectively, using GSH (Figure 7).

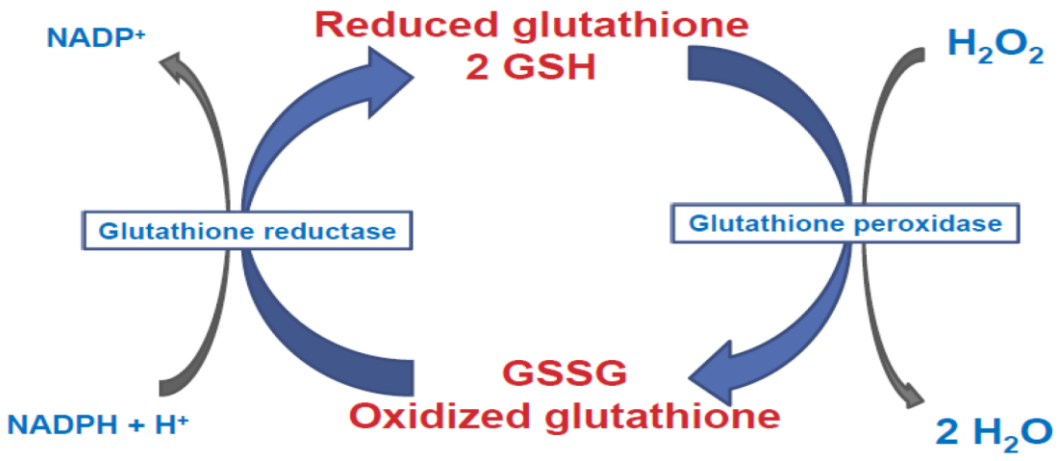

Figure 7. Reduction of peroxides by GPx using GSH.

This reaction leads to the accumulation of GSSG that causes a shift to the ratio of GSSG to GSH leading to more oxidized redox potential. The redox potential of GSH is related to GSSG and GSH concentrations according to the Nernest equation: $\Delta E = \Delta E^{\circ} \cdot (RT/nF) \cdot \log([GSH]^2/[GSSG])$ where R is the universal gas constant, T is temperature in kelvin and F is the Faraday constant. At room temperature (25 °C), RT/F may be treated as a constant and replaced by 25.693 mV for cells (231). The role of redox potential in different cell functions and in systematic responses is discussed in the section 1.4.3.

Another important function of GSH is the detoxification of xenobiotics and their metabolites. These compound form conjugates with GSH either enzymatically (catalyzed by GSH-S-transferases) or spontaneously (225). These conjugates can be excreted out of the cells for example into bile (as in case of hepatocytes) or can go through the mercapturic pathway. It should be noted that GSH conjugation irreversibly consume GSH with no further recycling compared to the antioxidant function (226).

The third important function of GSH is the maintenance of essential thiol status. Being the most dominant non-protein thiol in the cell, GSH is essential to maintain the protein thiol status and the intracellular redox. In this reaction catalyzed by thiol-transferase, GSH undergoes thiol-disulfide exchange: GSH + protein-SSG ⇌ GSSG + protein-SH. As this is a reversible reaction so its equilibrium is determined by the cell redox potential (232). Under normal conditions, cellular GSSG concentration is kept very low (around 1%) to limit the formation of protein mixed sulfide. The importance of this thiol-disulfide equilibrium is that it regulates many metabolic processes like enzyme activity, signal transduction, gene expression via redox sensitive transcription factors such as nuclear factor kappa B and activator protein-1 (232-234).

The GSH role in the control of cell cycle is one of its very important functions. In lymphocytes, fibroblasts and rat hepatocytes, the early proliferative response was associated with significant increase in GSH level. This phenomenon was confirmed in cases with partial hepatectomy where the increase in GSH preceded the onset of increased DNA synthesis for proliferation and regeneration (235). In addition, blocking this increase in GSH leads to impairment of hepatic regeneration after partial hepatectomy (236). On the other hand, apoptosis was also linked to GSH; as GSH depletion was associated with apoptosis in many different cells even when initiated by an agent that is not an oxidant (237). Blocking GSH efflux from certain cells (U937 and HepG2 cells) prevented puromycin-induced apoptosis (238). The mechanism through which GSH exercise this function is thought to be through the regulation of the redox state of specific thiol residues of proteins like nuclear factor kappa B and caspases that are involved in cell death (239).

Lastly, cysteine is very unstable and can rapidly auto-oxidize to cystine if not incorporated in GSH molecule. Serving as a continuous cysteine source via the gamma-glutamyl cycle is one of the main functions of GSH (Figure 6) (240, 241).

1.4.3 Oxidant-antioxidant imbalance: macromolecular injury and redox biology

Under normal physiological conditions there is a balance between the production and elimination of oxidants. This balance is kept through the living organism's finely regulated system (242). One example of a successful maintenance of this balance is the upregulation of antioxidants short time before term birth to counteract the oxidant transition from the antenatal relatively hypoxic intrauterine life to neonatal life (Figure 5) (212, 243-247). Different situations can lead to failure to maintain this balance and these situations include:

- Increased exogenous or endogenous oxidant load.
- Decreased non-enzymatic antioxidants.
- Decrease or inactivation of antioxidant enzymes.

In certain situations, more than one of these factors could contribute to this imbalance which is the case in premature infants. While being exposed to highly increased oxidant load (supplemental oxygen, peroxides contamination PN, and infection) they suffer from precarious antioxidant defenses as well (Figure 8) (248-250). This imbalance can certainly affect almost all living processes as it will be discussed in the next section.

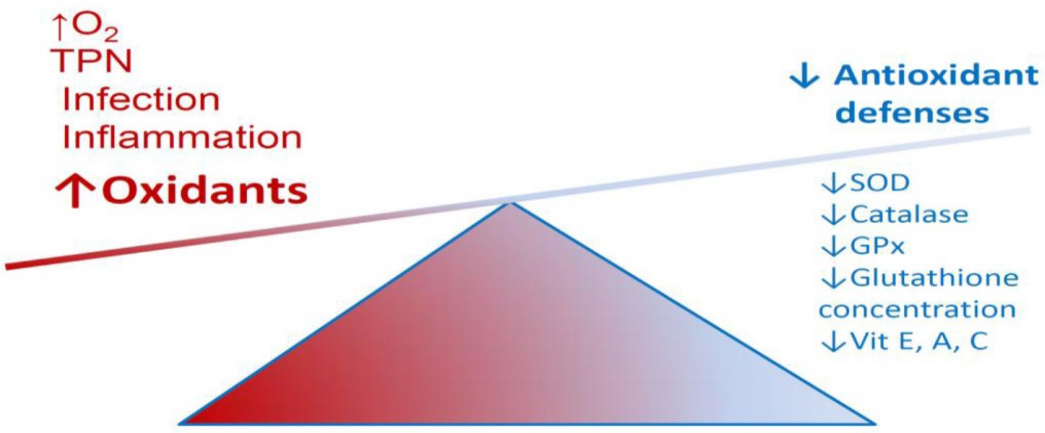

Figure 8. Imbalance between oxidants and antioxidants in preterm infants.

1.4.3.1 Oxidants and macromolecular injury

The oxidative mechanism classically linked to free radicals is the macromolecular oxidative damage. Free radicals are small molecules that have an unpaired electron (242). They are diffusible and tend to be reactive, so they can precipitate a chain reaction where a single free radical initiation can propagate and damage multiple molecules (242). The targets of macromolecular oxidative damage are lipids, proteins and DNA (251). In purified chemical systems, studies of chain reaction demonstrated that a single initiation event can damage up to 400 lipid molecules by oxidation to lipid peroxides before termination of the reaction sequence (186). At the termination of the reaction a lipophilic antioxidant (example vitamin E) will donate a hydrogen atom and form the non-radical products. The resulting lipid peroxides undergo decomposition to aldehydes such as malondialdehyde and 4-hydroxynonenal as specific end products (251). By the same coin, oxidation of proteins includes forming carbonyls and dityrosines while the DNA oxidation leads to 8-hydroxyguanine (8-OHG)

formation and other oxidized DNA bases. These resulting oxidation molecules are well reported and are classically used to evaluate oxidative stress (242).

For long time it was thought that these molecular damages adversely altering cellular functions to be the mechanism of oxidative stress effects. However, with more than 40 years of research on free radical chemistry and antioxidants in biological system resulted in an important evolution of our understanding. Now we know that antioxidant defenses including free radical scavenging enzymes as superoxide dismutase, the abundant radical scavenging non-enzymatic antioxidants as vitamin E and C and the very high protein concentration in biological system make free radical chain reaction almost completely preventable (186). One can conclude that in biological system, in contrast to purified system, free radical initiation events and not chain reactions are responsible of most free radical mediated oxidative damage. This distinction is very important in understanding oxidative stress as biological systems produce much more nonradical oxidants than free radicals (252). This advanced comprehension of the free radical biology and the fact that almost all large scale studies using free radical scavenging molecule as intervention showed no or little benefit both lead to a wide belief in the scientific community that there is other mechanisms that could explain oxidative stress effects without being directly related to macromolecular damage leading to the birth of the redox theory in the early two thousands.

1.4.3.2 Radical-free oxidative stress – Redox stress

Recent definitions of oxidative stress describe it as “An imbalance between oxidants and anti-oxidants, in favor of the oxidants, leading to molecular damage and/or a disruption of redox signaling and control” (186, 253). Free radical mechanisms in macromolecular damage

were the focus of many research studies. More recent studies focus more on the alternative mechanism involving non-radical 2-electron oxidants. Studies in biological system shows that univalent reduction of O_2 resulting in superoxide anion is always a small fraction of the bivalent reduction of O_2 resulting in H_2O_2 (Xanthine oxidase kinetic is one example) (252). In biological systems, free radical scavenging mechanisms (both enzymatic and non-enzymatic) will result in efficient conversion of the majority of the free radical to non-radical oxidants. Putting all this together will lead to the conclusion that free radical portion is minimal in biological system as depicted in (Figure 9) (186).

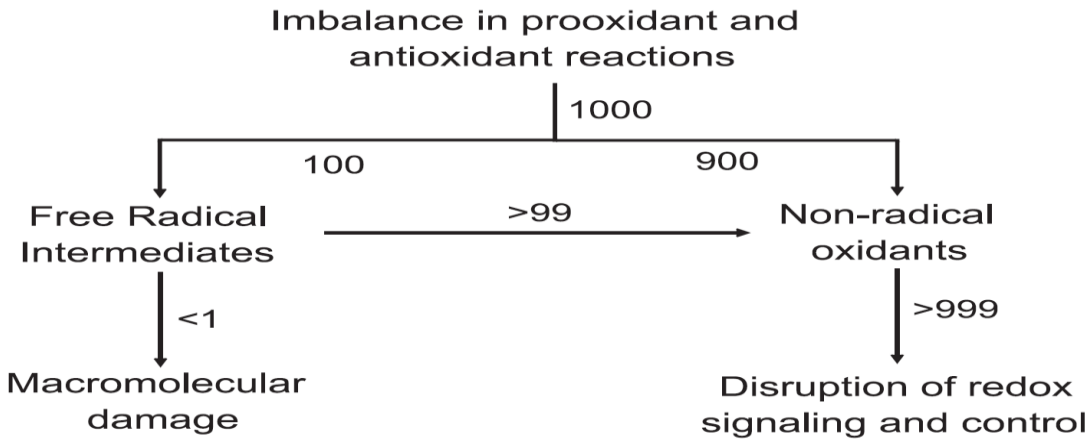

Figure 9. The two mechanisms of oxidative stress.

The interrelation between the two mechanisms of oxidative stress from (186). Published under Creative Commons (CC-BY) and do not require permission for reuse.

With this in mind, one can understand why if the abundant non-radical oxidant contributes to the pathology of a disease by redox signaling and control mechanism, the pathology can still correlate with the minimal free radical caused macromolecular damage, yet it will not respond

to free radical scavengers' treatment (186, 253, 254). These 2 faces of reactive oxygen species as oxidative stress and redox biology is depicted in (Figure 9) (186).

The non-radical oxidants can cause oxidative stress by perturbing the thiol redox circuits which control cell signaling and physiological regulation and could be termed redox stress (186). Here, I briefly describe the cysteine (Cys) oxidation as an example of the activation of H_2O_2 signaling pathway. Cys residues are known to be functionally active and were found to be over-represented in functional regions of proteins (255). Cys (SH) can be oxidized by low level H_2O_2 initially to sulfenic acid (SOH). Sulfenic acid is very reactive that rapidly forms a disulfide bond or sulfonamide in the presence of any thiol or nitrogen available respectively. (SOH) may be further oxidized with high doses H_2O_2 to sulfinic (SO_2H) or sulfonic (SO_3H) acid. To insure reliable cellular signaling these oxidized thiols should be reversible to inactivate the response after performing its function. This recycling of sensors should be slow enough to allow them to perform the intended function, but it also needs to be fast once started. To complete this part of sensors criteria, two systems of reduction exist, the thioredoxin and the GSH/Glutaredoxin system. It should be noted that (SOH), disulfide, and sulfonamide forms are readily reduced to the initial (SH) through these two systems. The (SO_2H) can be occasionally reduced by sulfiredoxin in an energy consuming process while (SO_3H) is not reversible in biological systems. This oxidation/reduction is the on/off switch for the signaling pathways according to H_2O_2 concentration provides the cell with a mechanism of sensing, responding and terminating the response to redox environment changes. This whole oxidation and reduction processes are depicted in (Figure 10) (256).

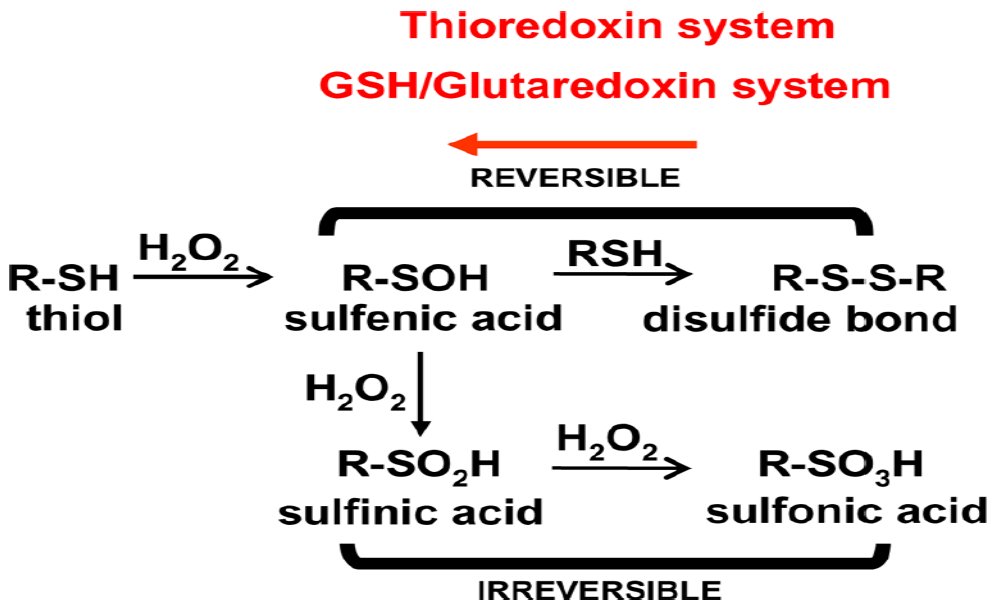

Figure 10. Different oxidation states of a sulfur atom upon H_2O_2-dependent oxidation.

One of the well-studied examples of H_2O_2 activated transcription systems is the H_2O_2 dependent activation of nuclear factor erythroid 2-related factor (Nrf2). Nrf2 plays an important role in the regulation of oxidative stress response. Its genetic deletion renders the animal and single cells much more prone to suffer from the damaging effects of oxidants and inflammatory agents (257-260), while its induction allows survival and adaptation under various conditions of stress (261). H_2O_2 is known to be able to activate Nrf2-dependent gene expression including oxidative stress response and detoxification pathways (262). In untreated cells, Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap) where it is anchored to it facilitating its ubiquitination. Upon treatment with 200 µM H_2O_2 transient oxidation of Keap1 molecules with creation of intermolecular disulfide bond linking 2 Keap1 molecules resulting in Nrf2 stabilization and migration to the nucleus where it forms a heterodimer with a small musculoaponeurotic fibrosarcoma (sMaf) protein that interacts with

Antioxidant response elements (AREs) and induces transcription of a genes encoding antioxidant response as shown in (Figure 11) (263-265).

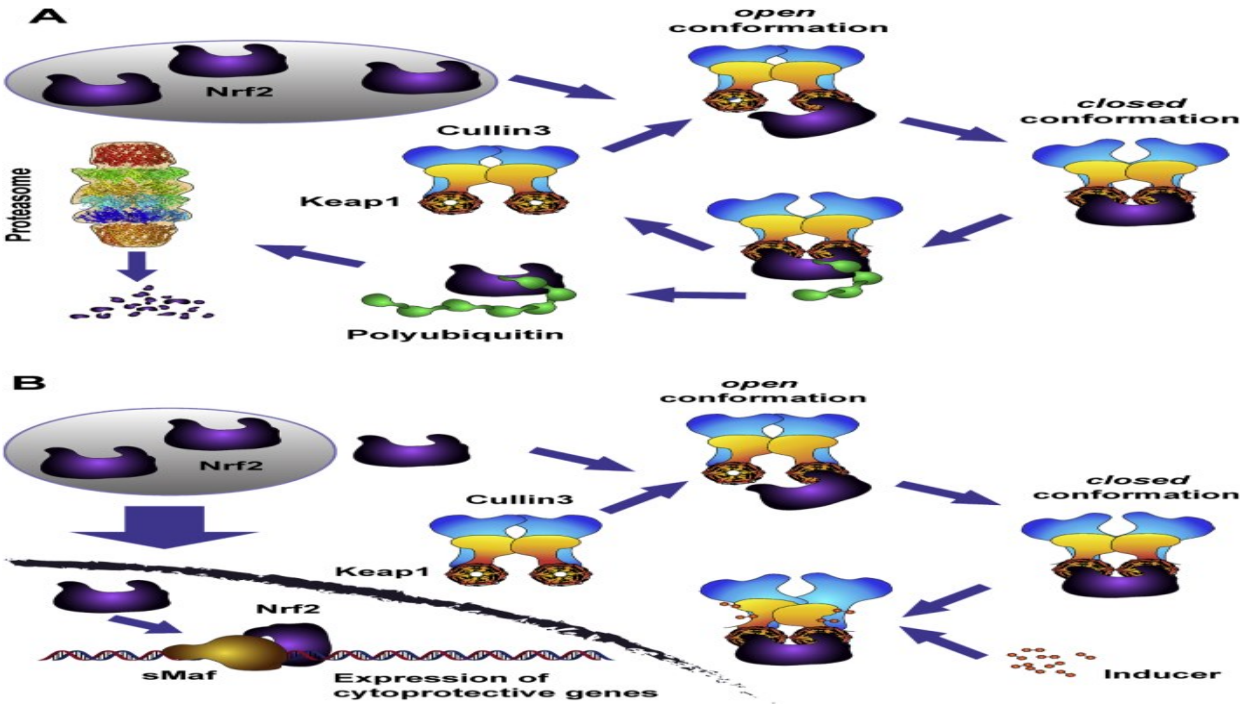

Figure 11. Inducers (oxidants and electrophiles) block the cycle of Keap1-mediated degradation of Nrf2

(A) At homeostatic conditions, Keap1 uses a cyclical mechanism to target Nrf2 for ubiquitination and degradation. (B) The cycle of Keap1-mediated degradation of Nrf2 is blocked by chemically modifying cysteine sensors of Keap1 and disabling its substrate adaptor function, leading to accumulation of the protein complex in the *closed* conformation (263). Open access under a Creative Commons License.

The oxidation of Keap1 by H_2O_2 behaves exactly as expected for a signaling pathway by being very transient and reversible. The process peaks about 5 minutes after treatment then fades. The recycling of the cysteine back to its reduced state is responsible for the reversibility

of this stimulation. This recycling is mediated among others by the GSH/Glutaredoxin and the thioredoxine systems. After discussing both the macromolecular damage and the redox stress responses it should be noted that various degrees of oxidative stress result in different responses and that is why it is important to discuss this intensity-based classification of oxidative stress.

1.4.3.3 Classification of oxidative stress

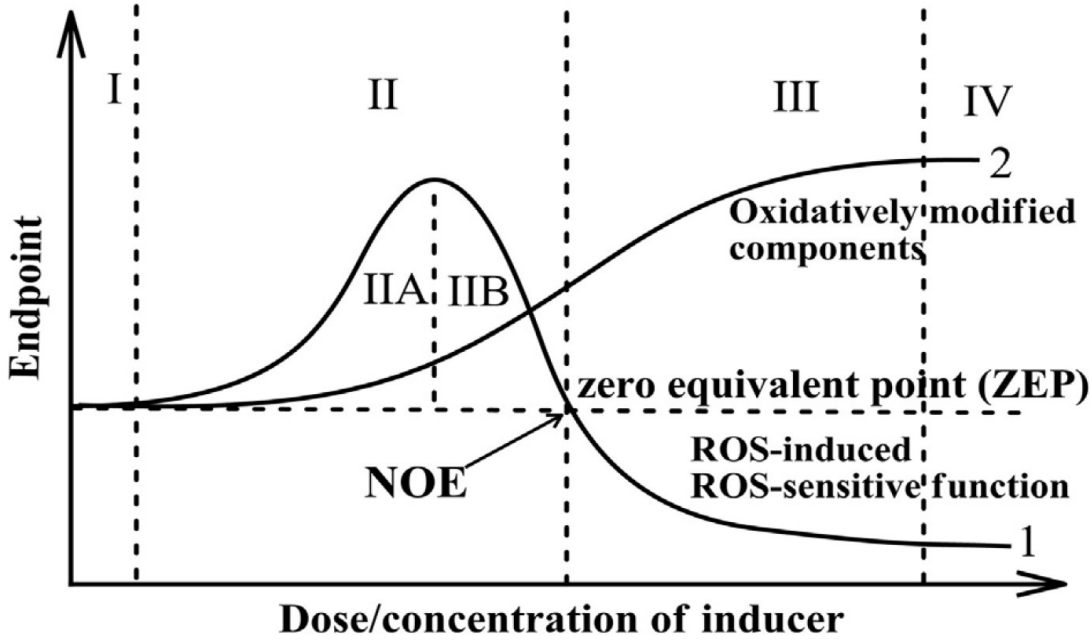

Figure 12. Schematic classification of oxidative stress.

This figure shows four zones proposed as: I – basal oxidative stress zone; II – low intensity oxidative stress; III – intermediate intensity oxidative stress; and IV – high intensity oxidative stress (242). Reuse license # 4335631491538.

Living organisms continuously generate ROS and they can successfully cope with them with a baseline antioxidant activity. This is called the basal oxidative stress zone (Figure 12. I). Low oxidative stress is induced with relatively small increase in ROS. This leads an increase in both the oxidatively modified molecules (Figure 12, curve 2) and the ROS signaling effects like increasing ROS induced parameters as antioxidant enzymes (Figure 12. curve 1). It should be noted that during low oxidative stress the curve I can have two zones: initial increase (IIA) and then decrease (IIB) after reaching its maximum while the increase of inducer continues. This decrease can bring curve I back to its baseline to reach the non-observable effect point. Further increase in the inducer will result in increasing the level of oxidatively modified molecules, while the ROS induced components will continue to decrease (intermediate oxidative stress, III). This decrease is due to degradation of the enzyme and /or ROS-inactivation of enzyme synthesis. With more increase in ROS these 2 curves reach plateaus which represent maximum saturation and indicate that all available substrates are already oxidized (high oxidative stress, IV). Obviously, this classification has its own limitations and exceptions. For example, if antioxidant capacity is already increased with preconditioning like physical training, this may result in a shift to the right of these curves. It is also known that different ROS induced components can be induced at different intensities of oxidative stress as a part of hierarchy of oxidative stress responses in animals (242, 253). In low oxidative stress states, mostly Nrf2/Keap1 system will be activated leading to adaptive response with induction of appropriate antioxidants. In addition to Nrf2/Keap1 system, Intermediate oxidative stress is known to induce the nuclear factor-kappa B (NF-κB) that promotes inflammation and a combined adaptive response and cellular damage will result. High oxidative stress is more likely to be associated with caspases activation leading to

apoptosis or with necrosis (242) . Recently these different levels of oxidative/redox stress were repeatedly shown to be linked to the cell cycle so in the next section we will describe this important relation and its effect on tissue remodeling (266-269).

1.4.3.4 Redox biology, cell cycle and tissue remodeling

GSH is the principal cellular redox buffer and its redox value was shown to be tightly connected to the cell biological state (231, 270). The redox potential turns molecular switches on and off for many metabolic pathways inducing cell proliferation, differentiation and apoptosis (254, 270-273). Proliferation occurs in more reduced cells and is accompanied by high metabolic rates leading to an increase ROS production, oxidation of the redox potential and slowing of the proliferation rates (200, 231, 273). In mildly oxidized redox potential environment, differentiation will take place. If oxidation of the redox potential continues cells will react either by inducing activation of redox sensitive factors leading to increase GSH production, reduction of redox potential and starting a new cell cycle with more proliferation, or if the oxidation of redox potential could not be counteracted, cells my undergo apoptosis and even necrosis in more severe cases (231, 273). This interrelation between cell redox state and cell cycle is depicted in (Figure 13) (200).

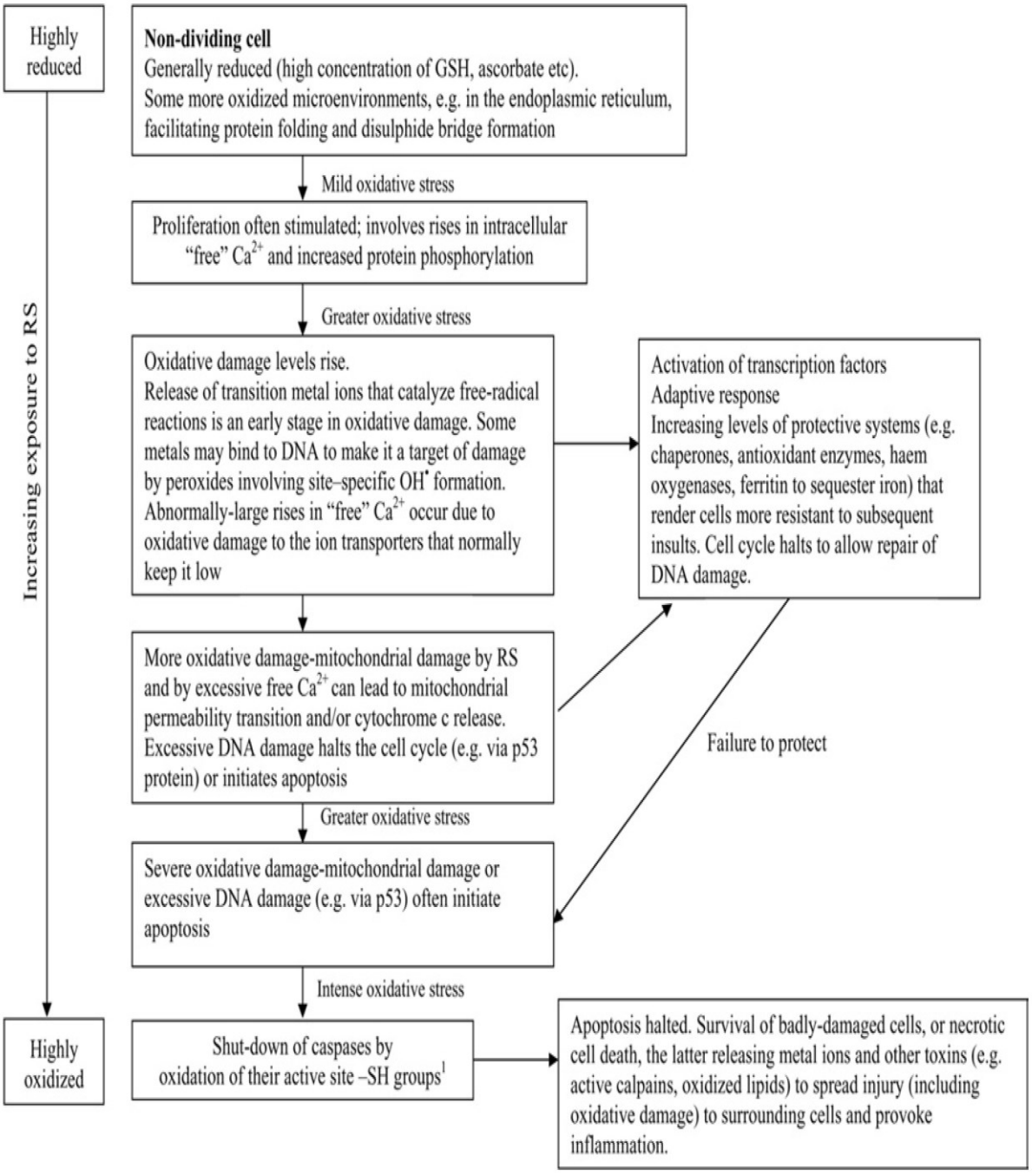

Figure 13. Cellular response to increasing exposure to reactive species (RS)

With increasing oxidative stress from more reduced to more oxidized cells go through the cell cycle from quiescence to apoptosis (200). Reuse permission is not required.

Elegant works have proved that this concurrence between redox potential status and cell cycle is not an association but rather a cause and effect relation. One of such well-designed works described the role of change in redox state in regulation of cell proliferation (232). In this study at the baseline proliferating fibroblasts were more reduced -34 mV than confluent, contact-inhibited cells. Treatment with GSH synthesis inhibitors resulted in more oxidized redox potential and significantly decreased proliferation. Treatment with GSH substrates resulted in more reduced redox potential and significantly increased proliferation (232). Studies in many tissues and different species suggested certain redox to correlate with certain cell cycle stages (274-276). It is also to be noted that this change in redox potential represents a continuum and what can be considered as a little redox potential change can have great impact on cellular activity. For example, treatment with 0.05 m*M* n-acetyl-cysteine resulted in more reduced redox potential by only -8 mV 24 hours after the end of this treatment. This small change in redox potential lead to as high as 26% increase in culture density compared to control (232).

Pulmonary development is a continuous process that needs cell proliferation, differentiation, and controlled apoptotic events. The lung cells must pass through these various cell cycle stages to permit continued remodeling (45, 92, 268). In an actively developing organ, an oxidized redox potential will decrease proliferation and favours apoptosis leading to arrest of development (231, 273, 277) and that is how an oxidized redox potential can be directly linked to the pulmonary arrest of development that characterizes BPD. The most known risk factor for BPD is oxygen toxicity can be explained from the redox biology point of view. The superoxide load resulting from high FiO_2 exposure is dismutated by SOD to H_2O_2 that results in more oxidized redox potential (186). Another source of peroxides toxicity in the neonatal

period, however less known, is the PN contamination with peroxides (1, 4, 278). In the following section I will discuss the PN contamination with peroxides and its possible role in BPD development.

1.5 Parenteral nutrition contamination with peroxides and its relation to BPD

Nutritional needs of the foetus are totally met through the placental supply. The fetal period is characterized by very rapid growth velocity and very high nutritional needs. A 27 weeks GA fetus (around 1100 g) will triple its weight in 13 weeks to reach normal full-term infant's weight (around 3300 g). Extremely preterm infants, while having these very high nutritional needs, are lacking fully functional digestive system and they usually need few days if not weeks before being fully fed. Advances in neonatal medicine led to marked increase in the survival of extremely preterm infants from about 10% survival of 28 weeks GA infants in the late 80s to the current survival rate of more than 90%. Concurrent advances in clinical nutrition permitted the development of PN that helped to partially meet extremely preterm infants' high nutritional demand whenever preterm infants are not able to fully meet their nutritional needs with enteral feeding. PN is composed of the 3 major nutritional elements: carbohydrate in the form of glucose, proteins in the form of amino acids and lipids in the form of fatty acids. Electrolytes like sodium and potassium, calcium and phosphorus as well as microelements are added to the glucose and amino acid moiety of the PN. To supply preterm infants with the necessary vitamins a multivitamin preparation is usually added to the glucose amino acid moiety. In some practices the glucose and amino acid moiety is mixed with the lipid moiety in what is known as all in one preparation of PN. The preparation of PN is

performed in sterile condition under ambient air environment. The presence of strong oxidants like O_2 and strong reducers as ascorbic acid, lipids and amino acid leads to chemical interactions resulting in the production of peroxides and other molecules that inevitably contaminate all PN solutions.

1.5.1 Peroxides contaminating PN and its sources

Since early nineties multiple studies confirmed the contamination of PN constituents with peroxides in clinically relevant solutions (279-283). The study by Lavoie et al was the first to quantify the total peroxide load received by preterm infant on PN and specify the contribution of each component (1). To the surprise of many, multivitamins solution (MVI) was found to be the principal source of peroxide contamination (1). The addition of the MVI to lipid free PN solution increased the amount of peroxides by 10 times while it increased it four times in the lipid containing PN solution. Quantitively, the main peroxide contaminating PN was found to be H_2O_2 and it represented 88% of peroxide contamination (1). In addition, a dose response increase of peroxide with the increase of multivitamins concentration was confirmed (1). This was explained by the fact that the photo-excitable riboflavin catalyses the oxidation of electron donors such as amino acids, lipids, and ascorbate by oxygen producing hydrogen peroxide in this light-dependent reaction (3, 4, 278). Photoprotection of the PN solution resulted in significant reduction of contaminating peroxides (146±15 vs 215±24 mM equivalents tert-butyl hydroperoxide (1). Another important factor contributing to peroxide contamination was the contact with O_2 in the air during compounding PN so protection from air was also tried but photoprotection was found to be more feasible and efficient (4). This photoprotection was achieved via covering PN with opaque bags and using orange or yellow

tubing with special emphasis that this photoprotection should start from compounding through intravenous delivery (4, 284).

1.5.2 *In vitro* effects of PN peroxide contamination

At the same or lower concentration of peroxides as reported in the PN, multiple toxic effects were documented both in cellular and tissue model. At cellular level, peroxides decreased endothelial cells viability (285), intracellular ATP content (286-288) as well as the synthesis of phosphatidyl by pneumocytes type II (286) and eicosanoid in endothelial cells (289). In addition, it leads to energy depletion and increased apoptosis in neonatal cardiomyocytes (290, 291). It should be noted that these effects were in some studies dependent on GSH level that was in turn related to the gender of studied cells (285).

In vitro isolated organs, similar concentrations of peroxides resulted in decreased myocardial contractility (292) and increased pulmonary vascular permeability (293, 294). These finding rose the question of what the effect of a similar peroxide contamination in a whole animal with competent antioxidant system will be.

1.5.3 Effects of PN peroxide contamination in animal model

1.5.3.1 PN peroxide contamination effects on the lungs

The first model of long-term infusion of parenteral nutrition for up to 25 days was already described since mid-eighties in guinea pigs (295). The purpose of this study was to evaluate the long-term effect of PN on growth and on the liver. This PN infusion model represented an ideal opportunity to study the effect of peroxide contaminating PN on different organs in a whole animal model. The effects of PN contamination with peroxides was initially

tested on the guinea pig lungs (296). The first study comparing controls, H_2O_2 injection, MVI with and without PN documented an oxidant effect on the lung of both H_2O_2 and PN with MVI on the lung as indicated by the changes in total GSH and oxidant sensitive eicosanoids (296). Peroxidation is known to induce procollagen gens expression, this was documented with PN infusion as well (297). This effect was reversed with the photoprotection of PN (297). In this model infusion of the light exposed-MVP induced a response on the procollagen mRNA similar to that of the infusion of 500 μMH_2O_2. The presence of this peroxide contamination affected the alveolar development with hypo-alveolarization (main feature of BPD) documented with light exposed PN or MVI (13). The effect of MVI on lung alveolarization could be because of H_2O_2 or photo exposure. While this effect was found with relatively high levels of H_2O_2 contamination (500 μM) which is equivalent to 2% concentration of MVI, it was not present with the concentrations more relevant to usual clinical PN infusion at around 1% concentration of MVI (around 250 μM) (13). This effect was reproduced in the presence of riboflavin and vitamin C only indicating that a light-induced by-product of vitamin C in multivitamin solution in the presence of riboflavin to be responsible of this effect (13, 16). This new molecule -by product-2,3-diketo-4-hydoxyperoxyl-5,6-dihydroxyhexanoic acid has both peroxide and aldehyde effects. It was later identified as ascorbylperoxide (AscOOH). AscOOH was shown to be associated with hypo-alveolarization and increased apoptosis guinea pigs' lung (13).

1.5.3.2 The PN contamination on the liver

To test the effect of PN contamination on the liver, the same guinea pig model was used. Results indicated prooxidant effects on the liver with H_2O_2 infusion in addition to

prooxidant with some antioxidant properties of PN with MVI (298). Functionally, both MVI alone and whole PN induced hepatic steatosis while simple infusion of H_2O_2 was not significantly different from control (299). The biologically beneficial effect of photo-protection was confirmed for again as the infusion of photo-protected MVI and whole PN significantly reduced hepatic steatosis suggesting that some MVI or PN component may become hepatotoxic after photo-exposure (299).

1.5.3.3 Metabolic effects of PN contamination with peroxides

PN contaminated with peroxides was also shown to have some short-term metabolic effects in the guinea pig model. The previously described PN specific peroxide (AscOOH) was shown to be associated with increased hepatic acetyl-CoA carboxylase enzyme activity (15). Increased glycolysis and increased lipogenesis were also associated with AscOOH infusion in guinea pig model (14). PN was also found to have a significant inhibitory effect on methionine adenosyl transferase (MAT) that controls the conversion of methionine into cysteine (the limiting amino acid for GSH synthesis) (18). This inhibitory effect resulted in low GSH level with more oxidized redox potential in the liver and the blood of guinea pig neonates (18).

Long-term impact of peroxide contamination of PN was first evaluated in the work of Kleiber et al that studied the effects of early neonatal PN infusion for 4 days on the metabolism of 12-14 weeks old guinea pigs (300). In this study, animals who received H_2O_2 or PN in early life demonstrated low body weight, low glucose tolerance and decreased spontaneous activity. This study raised the concern of possible long-term programming effects of PN contamination with peroxides that can last beyond that neonatal period and active PN exposure (300).

These multiple deleterious effects of PN contamination with peroxides led to active evaluation of the effects of this contamination in preterm infants with multiple significant results that I will summarize in the next section.

1.5.4 Effects of PN peroxide contamination in preterm infants

While it has been long time that the *in vitro* and animal model showed side effects of PN peroxide contamination. These effects started to be studies in preterm infants more recently (8). Few reasons make these studies more challenging, including the absence of preterm infants not exposed to PN (absolute control) and the difficulty of isolating the PN contamination effect off the many other oxidants to which preterm infants are usually exposed early in their lives. The model of randomized controlled study using photoprotection from light (that leads to less peroxide contamination) as a relative control to study of the complications related to PN contamination with peroxides in preterm infants was first described by Khashu et all in 2006 (8). Due to their vasoactive activities; peroxides contaminating the PN were hypothesized to favorize mesenteric vasoconstriction and lead to enteral feeding progression difficulty. To study the effect of decreasing these peroxides in the PN, a randomised controlled study with an intervention group receiving photo protected PN was completed. PN protection from light led to more increase in daily enteral feeds during the first 7 days of life leading to faster advancement of minimal enteral feeding (8). A secondary analysis of this study results showed higher blood glucose levels in infants receiving the light exposed PN and faster increase in plasmatic triglyceride concentration with the increase in lipid intake in the same group (7).

The pulmonary positive effects of decreasing PN contamination with peroxides was first suggested by a post-hoc analysis of pulmonary outcome in infants who participated in Khashu study (6). In this analysis a reduction of 30% in BPD or death was achieved in infants receiving PN protected from light. This decrease did not reach statistical significance, but its probability was 80% (6). Soon after, another group confirmed this positive effect of photoprotection on the prevention of BPD with statistically significant comparable decrease in lung disease (about 25%) in infants receiving light protected PN (147). In a study comparing the effects of photoprotection of PN and different coadministration of MVI to lipid moiety on pulmonary outcome, Chessex et al were able to demonstrate that early redox stress (on day 7 and 10 of life) correlates with the severity of BPD (146). In this study, infants who had more oxidized redox potential on day 7 of life had the more severe form of BPD at 36 weeks corrected age (Figure 14) (146).

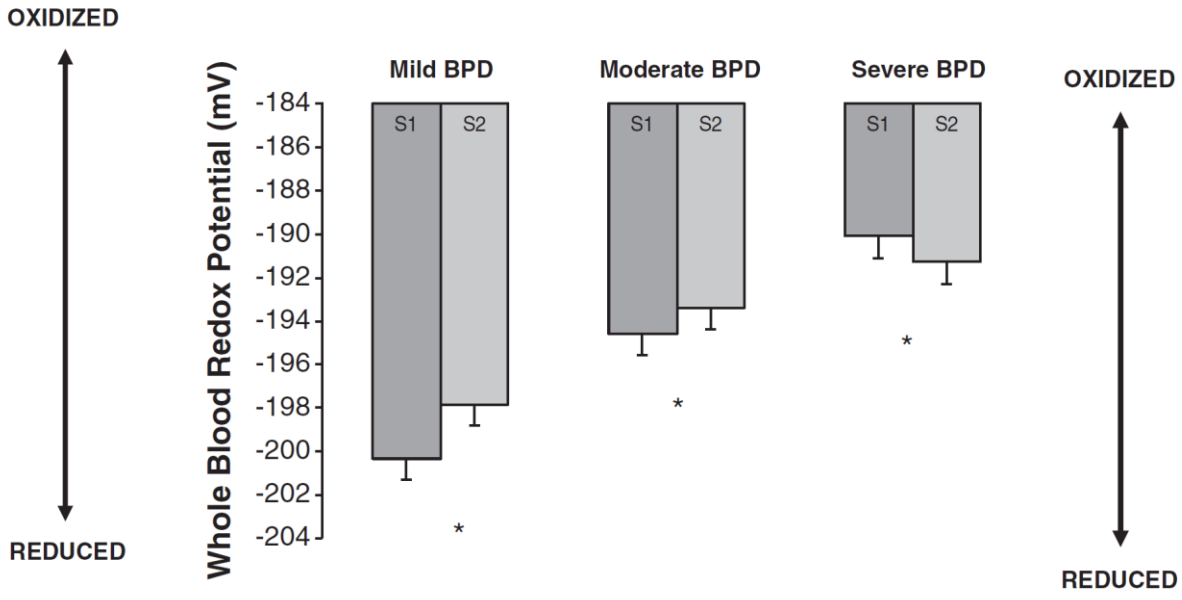

Figure 14. The relation between the redox potential on day 7 and 10 of life and BPD.

The redox potential of glutathione (mV) measured in whole blood on day 7 of life (S1) and day 10 (±24 h) of life (S2) was significantly more oxidized in infants who went on to develop mild (n=15) vs moderate BPD (n=20; *p<0.005) vs severe BPD (n=17; *p<0.001). Results are expressed as means±SEM (146). Reuse license # 4335981081085.

1.6 **Thesis rational, hypothesis and specific objectives:**

The studies of PN contamination effects on preterm infants, while they are strongly suggesting a relation between peroxides contamination of PN and BPD, they raised few questions that stimulated this work. These questions include: if the relation between PN contamination with peroxides and BPD is caused by the redox stress, is this redox stress momentary during the period when infants are receiving PN or does it extend beyond this period? What is the relation between the oxidant effect of PN contamination and other oxidants to which preterm infants exposed early in life as O_2 supplementation, blood transfusion and neonatal infections? If preterm infants are unable to detoxify the peroxides contaminating the PN, is this related to deficient glutathione as demonstrated in our guinea pig model, is possible to quantify a specific PN peroxide (AscOOH) in the urine of preterm infants and what can be the clinical relevance of such peroxide? The answers to these questions could help establishing new approaches that permit early identification of infants at higher risk and can even provide new clinical strategies that help decreasing the incidence of BPD.

In this work, our hypothesis is that in extremely preterm infants, peroxide contamination of PN in the early neonatal period leads to prolonged redox stress and contributes with other oxidants to the development of BPD. This effect of peroxide contamination is probably due to deficient detoxification of peroxides that leads to its accumulation in biological fluids and increase its concentration which can be used as an early marker of higher risk of BPD development.

Specific objectives:

- To outline the effect of early oxidant exposure (PN, O_2, infections and blood transfusions) on long-term redox stress status (at 36 weeks PMA).
- To outline the effect of early oxidant exposure (PN, O_2, infections and blood transfusions) on BPD.
- To explore the interaction between PN peroxide contamination and other major oxidants on the redox stress at 36 weeks PMA.
- To explore the interaction between PN peroxide contamination and other major oxidants on BPD.
- To test if the deficient detoxification of peroxides is related to glutathione deficiency as demonstrated in our guinea pig model. .
- To evaluate specific PN peroxide (AscOOH) as an early bio-marker of higher risk of BPD development.

2 Chapter 2: Oxygen and parenteral nutrition - two main oxidants - for extremely preterm infants: ‘it all adds up’

2.1 Complete reference, abstract and keywords

Mohamed I, Elremaly W, Rouleau T, Lavoie JC. **Oxygen and parenteral nutrition two main oxidants for extremely preterm infants: 'It all adds up'**. J Neonatal Perinatal Med. 2015;8(3):189-97

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- An abstract from this work was presented in the pediatric academic societies meeting, Vancouver, Canada, May 2014.

Short title: O_2 and PN association with oxidative stress and BPD

Authorship statement:

Ibrahim Mohamed: Dr. Mohamed conceptualized and designed the study, performed redox potential analyses, collected clinical data, carried out the initial analyses, drafted the initial manuscript, and approved the final manuscript as submitted.

Wesam Elremaly: Ms Elremaly contributed to the analysis and interpretation, reviewed and revised the manuscript, and approved the final manuscript as submitted.

Thérèse Rouleau: Ms. Rouleau coordinated and supervised the redox potential analysis, critically reviewed the manuscript, and approved the final manuscript as submitted.

Jean-Claude Lavoie: Dr Lavoie contributed to the study conception, the analysis and interpretation of the data. He critically revised the manuscript for important intellectual content and approved the final manuscript as submitted.

Abstract

Objectives: To assess the effect of early exposure to O_2 and parenteral nutrition (PN) on oxidative stress at 36 weeks postmenstrual age (PMA) and on bronchopulmonary dysplasia (BPD) in extremely preterm infants.

Study design: A prospective observational study including 116 infants < 29 weeks of gestation. Baseline clinical characteristics, FiO_2 on day 7, duration of PN and clinical outcomes data were collected. In 39 infants, whole blood glutathione (GSH) and oxidized glutathione (GSSG) at 36 weeks PMA were measured and the redox potential was calculated using Nernst equation. Student's t-test, Chi-square, Spearman correlation, ANOVA, and logistic regression analyses were used as appropriate. $P<0.05$ was considered significant.

Results: $FiO_2 \geq 25\%$ was associated with higher level of GSSG (0.29±0.04 versus 0.18±0.02 nmol/mg of protein), a more oxidized redox potential (-191±2 versus -198 ±2 mV) and more BPD (90% versus 45%). PN duration > 14 days was also associated with higher level of GSSG (0.26±0.03 versus 0.13±0.02 nmol/mg of protein), a more oxidized redox potential (-193±5 versus -203±2 mV) and more BPD (89% versus 24%). In logistic regression model, each 1% increase in FiO_2 and each day increase in PN duration resulted in an increase in the OR for BPD by1.57 (1.09 - 2.28) and 1.17 (1.03 - 1.33) respectively.

Conclusion: Early O_2 supplement and PN have additive effects that were associated with prolonged oxidative stress and increased risk of BPD. Strategies targeting judicious use of O_2 and decreasing the duration or developing a safer formulation of PN can be targeted to decrease BPD.

Key Words: Oxygen; Parenteral nutrition; redox potential of glutathione; bronchopulmonary dysplasia; chronic lung disease; newborn; and extremely preterm infants.

2.2 **Introduction:**

Bronchopulmonary dysplasia (BPD) is one of the most common morbidities in preterm infants and is the most common chronic lung disease in infancy (1, 2). In addition to short-term consequences like increased mortality and length of hospital stay, BPD also increases the risk of long-term pulmonary and neurodevelopmental complications (3, 4). The etiology of BPD appears to be multi-factorial, but the oxidative stress seems a common point (5-12). Certain clinical practices increase the oxidant load in preterm infants including both oxygen supplementation and parenteral nutrition (PN), which is contaminated with peroxides (11-13). Higher oxygen supplement increases the levels of reactive oxygen species production such as superoxide anion and H_2O_2 with increasing O_2 tension (14). Almost all preterm infants less than 29 weeks of gestation age will need PN due to their intestinal immaturity. The reduction (gain of electron) of dissolved oxygen in PN solution by polyunsaturated fatty acids, amino acids and vitamin C produces peroxides (13, 15, 16). During detoxification of peroxides by glutathione peroxidase, GSH is oxidized into GSSG. Due to glutathione abundance, the redox couple GSSG/2GSH conditions the cellular redox environment that is an important modulator of the cellular metabolism (17).

In preterm infants, short-term exposure to high FiO_2 during delivery room resuscitation is associated with both increased oxidative stress markers (blood GSSG/GSH ratio on day 3 and 7 of life) and risk of BPD (18). Oxidized redox potential of glutathione measured in blood of premature infants on day 7 of life is associated with severity of BPD (11). The frequent

associations between oxidative stress markers and BPD suggest that oxidative stress is a leading factor in the development of BPD (5, 7-12).

BPD is diagnosed at 36 weeks postmenstrual age (PMA), several weeks after initial PN and O_2 supplementation. We hypothesize that O_2 supplement and PN early in life could induce prolonged and additive oxidative stress, and thus increasing the risk of developing BPD. Therefore, the aim of this study was to assess the relation between O_2 and PN as main sources of oxidants and the redox potential status (GSH, GSSG, redox potential) measured at 36 weeks PMA and the incidence, and severity of BPD in infants < 29 weeks of gestational age.

2.3 **Methods:**

2.3.1 Patients

This is a prospective observational study including 116 infants < 29 weeks without major congenital malformations admitted to the neonatal intensive care unit (NICU) before 24 hours of life between August 2010 and July 2011. The flow of participants is shown in **Figure 15**. The study and the informed consent forms were approved by the Research Ethics Board of the CHU Sainte-Justine (registration number 2792).

2.3.2 Local practices

Local NICU practices for extremely preterm infants were 1) target O_2 saturation between 85 to 94 % for infants who need supplemental O_2 whereas 2) parenteral nutrition is prescribed in the first day of life at 80 ml/kg/day and increased 10-20 ml/kg/day to achieve 150 ± 10 ml/kg/day. Amino acids (TrophAmine®, B. Braun, Bethlehem, PA) are started on

day 1 of life at 2.5 g/kg/d then increased 0.5 g/kg/day to achieve 3.5 g/kg/day. Multi-12 pediatric (Sandoz, Boucherville, QC, Canada) was mixed with the amino acid moiety of PN. Infants less than 750 g received 1.5 ml /day of multivitamins while those infants more than 750 g received 2.5 ml/day. Lipids (Intralipid® 20%; Pharmacia Upjohn, Baie d'Urfé, QC, Canada) are started on the first day of life at 1-2 g/kg/day and increase by 0.5 g/kg/day to achieve 3 g/kg/day and administered separately. PN was not protected from light. Minimal enteral feeding was started at 20 ml/kg/day as soon as the medical condition of the infants was stabilized. If baby's mother chose to breast feed her infant, maternal breast milk was given to the baby once available during the first 3 days of life otherwise formula milk was started. This minimal enteral feeding was kept for 4 days and then a progressive daily increase of 20 ml/kg/day was prescribed if well tolerated by the infant. With these local guidelines, minimal time to reach full enteral feeding and PN duration was typically of 14 days (the defining criteria for the minimal PN exposure group).

2.3.3 Measurements

Glutathione measurements were obtained as followed: Within 4 min of sampling, 200 µL whole blood (EDTA tube) were mixed with 600 µL freshly prepared metaphosphoric acid (5%, w/v), centrifuged at 10000 rpm for one minute. Pellet (for protein assays) and supernatant were separated and stored at −80 °C. Whole blood levels of GSH and GSSG in supernatant fraction were measured by capillary electrophoresis (19). The whole blood redox potential (mV) was calculated using the Nernst equation (25 °C, pH 7.0) (17).

2.3.4 Definitions

Bronchopulmonary dysplasia was defined as the need for supplemental oxygen at 36 weeks (20, 21). BPD severity was determined using the National Institutes of Health Workshop severity-based diagnostic criteria (22).

The Score for Neonatal Acute Physiology, version II (SNAP- II), is a simplified neonatal illness severity score that measures six physiology - based items during a 12-hour period, including lowest blood pressure, lowest temperature, PO_2 /FiO_2 ratio, lowest serum pH, seizures, and urine output (23). This score was used to adjust for initial severity of illness.

2.3.5 Study groups and comparisons

The FiO_2 on day 7 of life was used as an indicator of early O_2 supplement. Minimal exposure was represented by FiO_2 less than 25% (11). The duration of PN was used as a proxy of the amount of peroxides contaminating the PN received by the infant. Because of local practices, minimal exposure to PN was defined as fourteen days of PN or less. Redox potential, GSH and GSSG in the whole blood at 36 weeks PMA as well as severity of BPD were compared between infants receiving minimal FiO_2 and PN and those with higher exposure to these oxidants.

2.3.6 Statistical analysis

Data were summarized as proportions, means with standard error of the mean (SEM), or median with 25th-75th percentiles. All analyses of the relations between early O_2 and PN and BPD in group II was limited to the development of BPD using traditional definition and

their relation to BPD severity was analyzed in group I due to sample size. The comparisons were performed using Chi-square, t-student test, Spearman correlation as appropriate or ANOVA after verification of homoscedasticity by the Bartlett's χ^2 test. In case of significant heterogeneity of variance, data were logarithmically transformed to meet the homoscedasticity. If ANOVA test was significant, a follow up test was done using Tukey's range test to identify significantly different groups. Because no mathematical transformation satisfied the Bartlett's χ^2 test for the comparison of FiO_2 values observed on day 7 of life in function of severity of BPD, the Chi-square test has been used. Logistic regression was used to determine the independent effect of variables. A $p < 0.05$ was considered statistically significant.

2.4 **Results:**

To investigate whether Group II was representative sample of Group I, we compared clinical baseline characteristics (**Table 1**). There was no statistical difference between groups.

At 36 weeks PMA, the redox potential was more oxidized and GSSG was higher in infants with $FiO_2 \geq 25$ on day 7 and PN > 14 days (**Table 2**). The smallest infants (GA and BW) received a greater FiO_2 ($p < 0.001$) and longer duration of PN ($p < 0.001$). However, the redox potential measured at 36 weeks PMA did not correlate (Spearman correlation) with neither GA ($p = 0.3$) nor BW ($p = 0.61$). **Figure 16** suggests that the oxidative stress effects of O_2 and of PN were additive. Infants with PN duration > 14days and $FiO_2 \geq 25\%$ had more oxidized redox potential then infants with PN duration > 14 days but $FiO_2 < 25\%$ and those infants with PN duration ≤ 14 days and $FiO_2 < 25\%$, ANOVA ($p = 0.03$). The group of infants on $FiO_2 \geq 25$

and having PN ≤ 14 days included only one infant (redox potential = -199.1 mV) and could not be included in the ANOVA.

Compared to infants on FiO_2< 25%, the severity of BPD increased ($p < 0.01$) in the $FiO_2 \geq$ 25% group (**Table 3**). The relation between BPD severity and the duration of PN is showed in (**Figure 17)**. Data from all infants (**Figure 17**) demonstrated that the duration of PN was longer in neonates with moderate BPD ($p < 0.01$) and longest with severe BPD ($p < 0.01$). (**Table 4**) suggests an additive effect of FiO_2 and PN on the development of BPD.

The FiO_2 and the duration of PN could be the intermediates by which the severity of infant's disease may cause BPD; thus, a logistic regression model was designed. Factors included in the BPD definition like supplemental O_2 duration and mechanical ventilation duration were excluded from the model. Other reported risk factors for BPD such as gestational age, birth weight, male sex, intrauterine growth restriction, maternal preeclampsia/eclampsia, chorioamnionitis, antenatal steroids, illness severity SNAP-II score, significant patent ductus arteriosus, nosocomial infection and blood transfusion were used initially in univariate analyses (24-27). Gestational age, birth weight, illness severity SNAP-II score, significant PDA, nosocomial infection and blood transfusion in addition to FiO_2 on day 7 and PN duration were associated with BPD in our cohort with P value ≤ 0.1 (**supplementary data in 2.10**). Considering the co-linearity between gestational age and birth weight; only gestational age was kept in the logistic regression model (**Table 5**), In this model for each 1% increase in FiO_2, OR (95% CI) for BPD was increased by 1.57 (1.09 - 2.28), ($p = 0.02$) and for each day increase in the duration of PN, the OR was increased by 1.17 (1.03 - 1.33), ($p < 0.02$) indicating an independent effect of these factors in the development of BPD.

2.5 **Discussion**

The main findings of our study are that 1) early exposure to $FiO_2 \geq 25\%$ and longer PN duration are associated with more oxidized redox potential of glutathione at 36 weeks of PMA, 2) the effect of O_2 and PN exposure on redox potential seems additive, 3) FiO_2 on day 7 of life and PN duration are associated with the development of BPD, 4) their effects on the development of BPD are additive and independent.

Oxidative stress effect of O_2 exposure early in life of preterm infants was described in the elegant works by Vento et al comparing resuscitation with low O_2 ($FiO_2 \leq 30\%$) versus high O_2 ($FiO_2 \geq 90\%$). The high O_2 group had evidence of increased GSSG/GSH ratio in blood on day 1 and day 3 of life in extremely preterm infants (18). Similar oxidative stress was documented for a longer period (up to 4 weeks following exposure) in near term infants (28). Our work confirms the oxidant effect of early exposure to oxygen, on day 7 of life, on the GSSG, GSH and the redox potential measured several weeks after exposure, at 36 weeks PMA, the time of BPD diagnosis.

This was a prospective study aiming to follow infants exposed to more oxidative stress early in life (either $FiO_2 \geq 25\%$ on day 7 of life or PN duration > 14 days or both) till 36 weeks PMA when the diagnosis of BPD is made. In these groups, we found more oxidized redox potential and higher rate of BPD. While O_2 supplement at 36 weeks PMA is a part of the definition of BPD and it could partially explain oxidized redox potential of glutathione in infants with BPD; this confounding does not explain why these infants had high FiO_2 or longer duration of PN (with absence of chronological relation). On the other hand, early exposure to oxidants can be the cause of abnormal lung development leading to BPD (19, 29-

32). Furthermore, our finding that the exposure to these oxidants is associated with additive effect on both the redox potential and BPD represent another argument that these findings are most probably produced by the accumulation of the previous exposures and are not only due to the concurrent exposure to O_2 at the time of diagnosis of BPD even if the temporal effect of O_2 on the redox potential cannot be completely excluded.

The fact that a similar impact on glutathione status at 36 weeks PMA occurred after high FiO_2 exposure and with infusion of PN more than 14 days suggests a common oxidative pathway. Because glutathione is affected, peroxides generated *in vivo* after high FiO_2 or infused with PN are strongly suspected. Indeed, the radical superoxide anion generated following high FiO_2 is rapidly transformed in H_2O_2 by superoxide dismutase. Many works emphasized the role of O_2 as a radical oxidant for preterm infants (8, 12, 33, 34). This work supports a complementary aspect by showing the oxidative effect of non-radical oxidant (like H_2O_2) from inspired oxygen or from contaminated PN, on an important prematurity related complication. Because both, $FiO_2 \geq 25\%$ on day 7 and PN > 14 days were associated with development of BPD, this suggests that modifications of redox potential by high FiO_2 or PN induce an early remodelling or a delay in lung development leading to the need a sustained oxygen supplement. The characteristic changes in BPD including arrest of alveolar and vascular development were reproduced in different animals' models using either the hyperoxia model (29, 30, 32) or the infusion with PN model (19, 31). In this cohort, early O_2 and PN led to an oxidized redox status and increased odds of BPD at 36 weeks PMA. A possible mechanism explaining this relation is that the redox potential is recognized to act as a switch for several metabolic pathways, including cellular proliferation, differentiation and apoptosis (17, 35).

Modifications of these pathways can result in the characteristic arrest of vascular and alveolar development that is well documented in BPD (36, 37).

In our population, whole blood redox potential was -194.7±1.7 which is oxidized compared to reported measures from adults' cohort of 12 postmenopausal women at risk of oxidant stress because of a BMI of 30–40 but without type 1 or 2 diabetes or any history of chronic inflammatory disease (- 209±1) (11). However, these results were in concordance with others obtained from infants less than 28 weeks GA (-193±1) (11). These results point out the exposure of these infants to marked oxidative stress with decreased antioxidant capacity. The long-term impact of this increased oxidative stress was examined in animal models where neonatal exposure to hyperoxia (FiO_2 80%), from day 3 to day 10 of life, lead in the adult rat to increased blood pressure (by an average of 15 mm Hg), vascular dysfunction (with increased maximal vasoconstriction and impaired endothelium dependent vasodilatation), microvascular rarefaction (with 30% decrease of capillary density) and reduced nephron number (by 25%) compared to rat in room air oxygen (38). In parenteral nutrition model, guinea pigs exposed to injected peroxides either with or without PN from day 3 to day 7 of life had lower body weight, lower glucose tolerance and lower physical activity with a phenotype of energy deficiency (39). These findings suggest that the frequently reported associations in humans between preterm birth and high blood pressure (40), high insulin resistance (41) and diabetes (42) could be related to the higher oxidative stress exposure of preterm infants.

In humans, the association between prolonged PN and BPD was previously documented by Ehrenkranz *et al.* and Wemhoner et al (29, 30). In Ehrenkranz's cohort (October 1999 - August 2001) infants with prolonged PN received significantly less total nutritional support during the first 3 weeks of life which could explain the increased incidence of moderate/severe

bronchopulmonary dysplasia. It has been suggested that the influence of critical illness on the risk of BPD was mediated by total daily energy intake during the first week of life (43). In Wemboner's cohort (August 2004 - December 2006) the same association was found, however, without any statistically significant differences in the total intake of fluids, calories, glucose or protein and weight gain per day in both groups (44). Wemhoner et al suggested a protective role of critical minimal amount of enteral feeding is required to prevent BPD (44). Our results suggest that the toxicity (oxidant load) of PN plays a major role in the pathogenesis of BPD. The fact that adequate photo protection of PN reduces by half the peroxide concentration in PN (15) and reduces incidence of BPD (45) or chronic lung diseases (10) also offer strong arguments in favor of this hypothesis.

Because more premature or sicker infant may require higher FiO_2 and longer PN supplementation, gestational age or the illness severity could potentially be confounding parameters (26, 46). However, the logistic regression model results indicate that the effects of O_2 and PN on BPD are independent. The additive effect of O_2 and PN on BPD development shown in (**Table 4**) supports their independence and the notion of a common pathway leading to BPD.

In this study, an observational prospective cohort design was used. This was the most appropriate design in human neonate context as the exposure to O_2 and PN should be guided by the current clinical guidelines. We did not photoprotect the PN in this study. The current clinically available methods of photoprotection are all partial photoprotection methods and hence confer no additional benefit to infants while consuming valuable resources (47). As a referral center, our unit policy is to transfer all stable infants to their local hospitals. This explains the noticeably small number in both no BPD and mild BPD categories and the

apparent increase in the incidence of BPD. Furthermore, to be able to perform severity of BPD analyses in relation to FiO_2 on day 7 of life, we combine these 2 categories (no BPD and mild BPD) together to get appropriate number for statistical analysis. A second limitation of this study is the lack of explaining mechanisms relating the oxidized redox potential to the development of BPD.

This study reports for the first time the oxidative stress status in extremely preterm infants at 36 weeks PMA. While the oxidative stress role of O_2 is well studied in both animal model and human, our results underline the important role of PN as a source of oxidants that can contribute to the development of BPD. Our results also suggest an additive effect of both O_2 and PN at the level of oxidative stress as well as BPD development. This leads us to speculate that implementing a clinically feasible and safer mode of PN administration or developing peroxides free PN could have a significant impact in reducing this major complication of prematurity.

2.6 **Conclusion:**

We conclude that early O_2 supplement as well as the PN, as currently compounded, are associated with prolonged oxidative stress and increased risk of BPD. Further studies should assess whether strategies favouring judicious use of O_2 supplement and safer PN therapy could help reduce the oxidative stress experienced by extremely premature infants and decrease the incidence of BPD.

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2.8 **Tables**

Table I. Baseline clinical characteristics of all eligible infants and blood sample consent infants

	All participating infants (n = 116)	**Blood sample Consent (n = 51)**
Gestational age, mean ± SEM (weeks)	$26^{3/7} \pm {}^{1/7}$	$26^{4/7} \pm {}^{1/7}$
Birth weight, mean ± SEM (gram)	867 ± 21	846 ± 23
Apgar score at 5 minutes[a]	6 (5 - 7)	6 (5 - 8)
SNAP - II score, mean ± SEM	19 ± 1	19 ± 2
Sex		
Female, n (%)	49 (42%)	21 (41%)
IUGR, n (%)	24 (21%)	8 (16%)
Vaginal delivery, n (%)	41 (35%)	17 (33%)
Antenatal steroid		
Complete course, n (%)	60 (52%)	26 (51%)
Incomplete course, n (%)	44 (38%)	23 (45%)
Suspected chorioamnionitis, n (%)	16 (14%)	8 (16%)
Maternal preeclampsia, n (%)	22 (19%)	7 (14%)
Maternal diabetes, n (%)	16 (14%)	7 (14%)

[a] Presented as median (25^{th} –75^{th} percentiles)

Table II. The effect of O_2 on days 7 of life and the duration of PN on GSH, GSSG and the redox potential measured at 36 weeks PMA:

	FiO_2 < 25% (n = 20)	**FiO_2 ≥ 25% (n = 19)**	**P**	**PN ≤ 14 days (n = 6)**	**PN > 14 days (n = 33)**	**P**
GSH[a]	7.6 (0.5)	7.4 (0.6)	0.79	7.5 (1.2)	7.5 (0.4)	0.99
GSSG[a]	0.18 (0.02)	0.29 (0.04)	0.03	0.13 (0.02)	0.26 (0.03)	0.04
Redox potential[b]	-198 (2)	-191 (2)	0.02	-203 (5)	-193 (2)	0.03

Mean (s.e.m.); [a]GSH, GSSG are in nmol/mg of protein, [b]Redox potential in mV.

Table III. Severity of BPD in function of FiO_2 measured on day 7 of life

	FiO_2 < 25%	**FiO_2 ≥ 25%**	**Total**
No and mild BPD	29 (88)	4 (12)	33
Moderate BPD	10 (56)	8 (44)	18
Severe BPD	15 (28)	30 (71)	45
Total	54	42	96

- The data are presented as the number of infants and its proportion (%). Severity of BPD was significantly ($p < 0.01$) dependent on the FiO_2 measured on day 7 of life.

Table IV. Effect of O_2 and PN on the incidence of BPD:

	PN ≤ 14days	**PN > 14days**	**Total**
FiO_2 < 25%	5/27 (19%)	21/27 (78%)	54
FiO_2 ≥ 25%	3/7 (43%)	35/35 (100%)	42
Total	34	62	96

The data are presented as the number and proportion of infants (%) in each group. The effect of FiO_2 and duration of PN on incidence of BPD were significant ($p < 0.01$). Results suggest an additive effect of O_2 and PN on development of BPD.

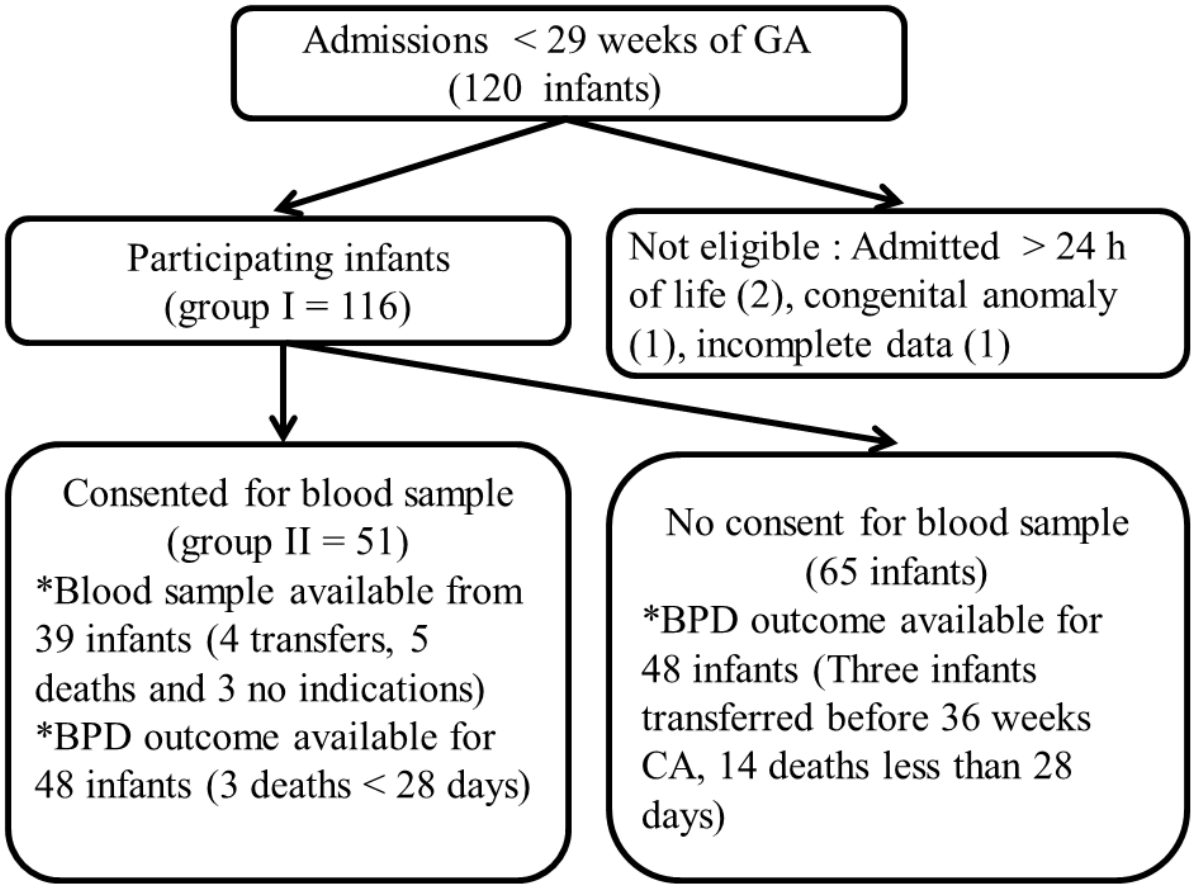
Table V. Logistic regression of risk factors of BPD

	Adjusted OR	95% CI	P Value
Gestational age	2.87	1.06 - 7.8	0.04
SNAP II score	1.16	1.02 - 1.31	0.02
Significant PDA	49.71	2.73 - 905	<0.01
Nosocomial infections	2.79	0.52 - 14.86	0.23
Blood transfusion	0.63	0.36 - 1.09	0.1
FiO_2 on day 7	1.57	1.09 - 2.28	0.02
Duration of PN (days)	1.17	1.03 - 1.33	0.02

- Significant PDA represents only treated PDA (either medical or surgical treatment), nosocomial infection represents the number of episodes of culture proven septicemia, urinary tract infection or meningitis, and blood transfusion represents the number of episodes of blood transfusion.

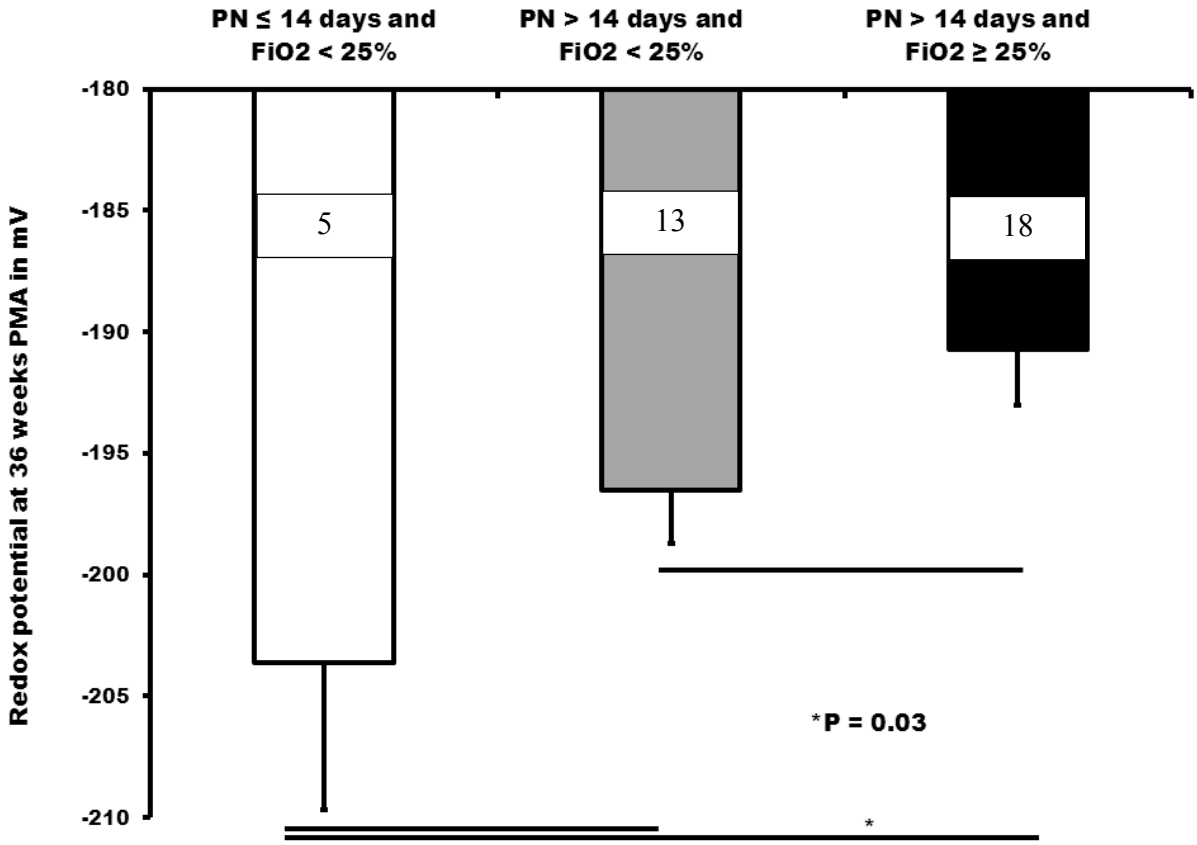
2.9 **Figures**

Figure 15. Participant flow.



Progress throughout the duration of the study, including flow of participants, transfer, early deaths and the numbers of infants with available redox potential results and BPD outcome results.

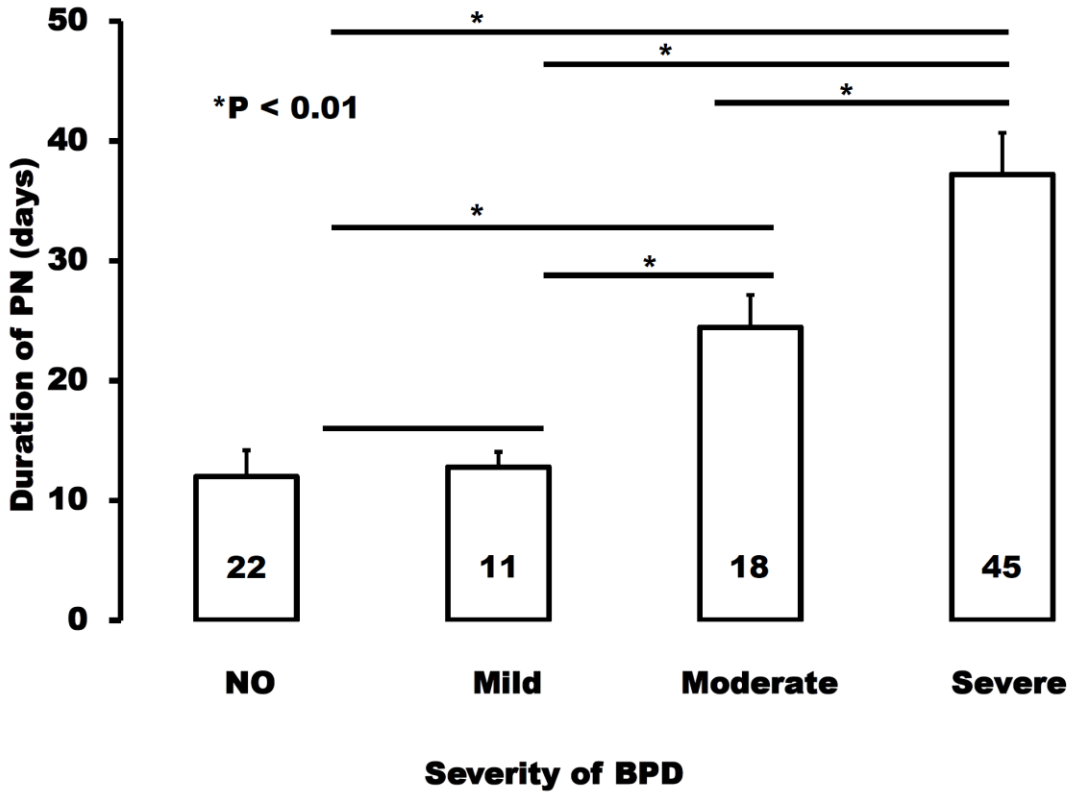
Figure 16. Effect of FiO_2 on day 7 of life and duration of PN on redox potential of glutathione at 36 weeks PMA.



Compared to the PN < 14 days and FiO_2 < 25% group, the redox potential was more oxidized in group PN > 14 days and FiO_2 < 25% while it was most oxidized in infants with FiO_2 ≥ 25% and PN > 14, suggesting an additive effect. (ANOVA, P = 0.03)

- Mean (±S.E.M.); n in each group is indicated within the column.

Figure 17. Relation between severity of BPD and duration of PN.



Increased duration of PN was associated with increased severity of BPD. (ANOVA, P < 0.01).

- Mean (±S.E.M.); n in each group is indicated within the column.

2.10 **Supplementary data:**

Comparison of demographic and clinical characteristics considered as risk factor of BPD between patients with and without BPD in this cohort.

Number of patients	BPD (n=63)	No BPD (n=33)	P value
Gestational age (W)*	26 $^{3/7}$ (22 $^{6/7}$ - 28 $^{6/7}$)	27 $^{3/7}$ (25 $^{4/7}$ - 28 $^{4/7}$)	<0.01
Birth weight (g)#	838 (±22)	1016 (±39)	<0.01
Male sex	39 (62%)	16 (48%)	0.21
IUGR	13 (21%)	4 (12%)	0.29
Preeclampsia/eclampsia	12 (19%)	4 (12%)	0.37
Chorioamnionitis	10 (16%)	2 (6%)	0.14
Antenatal steroids	34 (54%)	14 (42%)	0.49
SNAP-II score*	19 (9 - 25)	9 (0 - 9)	<0.01
Significant PDA	44 (70%)	2 (6%)	<0.01
Nosocomial infection*	1 (0 - 5)	1 (0 - 1)	<0.01
Blood transfusion*	5 (0 – 19)	2 (0 – 6)	<0.01

* Median (range), # Mean (±SEM), all other variables: absolute number (%).

- Chorioamnionitis refers to clinically suspected chorioamnionitis

- Significant PDA represents only treated PDA (either medical or surgical treatment)

- Nosocomial infection represents the number of episodes of culture proven septicemia, urinary tract infection or meningitis.

- Blood transfusion represents the number of episodes of blood transfusion.

3 Chapter 3: Ascorbylperoxide contaminating parenteral nutrition is associated with bronchopulmonary dysplasia or death in extremely preterm infants

3.1 Complete reference, abstract and keywords

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Ascorbylperoxide contaminating parenteral nutrition is associated with bronchopulmonary dysplasia or death in extremely preterm infants. JPEN J Parenter Enteral Nutr. 2017 Aug;41(6):1023-1029.

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Authorship statement:

I. Mohamed and J.-C. Lavoie had the idea for this study. I. Mohamed was responsible for collection of clinical samples and data, initiated the study protocol, and wrote the manuscript. J.-C. Lavoie had an important contribution in the understanding the biochemical mechanisms underlying the study; he revised the manuscript and led the team. W. Elremaly and T. Rouleau contributed to the study by their technical expertise; their advice for sampling and sample processing as well as chemical and biochemical determinations were essential; and they revised the manuscript before approving it. All authors approved the final version.

Abstract:

Background: Ascorbylperoxide (AscOOH) is hydrogen peroxide-dependent byproduct of ascorbic acid that contaminates parenteral nutrition (PN). It was shown to cause oxidized redox potential, increased apoptosis and decreased alveolarization in guinea pig model. Detoxification of AscOOH is carried out by glutathione peroxidase (GPx). We hypothesize that extremely preterm infants have limited capacity for AscOOH detoxification. Our objective was to determine if there is an association between early level of urinary AscOOH with later development of bronchopulmonary dysplasia (BPD) or death.

Materials and methods: A prospective cohort study including 51 infants < 29 weeks of gestation. Baseline clinical characteristics and clinical outcomes data were collected. Urine samples were collected on day 3, 5 and 7 of life for urinary AscOOH. Blood samples on day 7 were collected for total plasma glutathione, GPx and glutathione reductase (GR). Chi-square, t-student test, Spearman correlation (r), linear regression (adjusted r2) and repeated measure ANOVA were used as appropriate. $p< 0.05$ was considered significant.

Results: Urinary AscOOH increased overtime ($p=0.001$) and was higher in infants who later developed BPD or death outcome ($p=0.037$). Compared to adults and full term infants, total plasma glutathione concentration was low (1.02, 0.49-1.76 µmol/l: median, 25th –75th percentiles) whereas GPx and GR activities were sufficient (3.98±1.25 and 0.36± 0.01 nmol/min/mg prot. respectively).

Conclusion: Extremely preterm infants have low glutathione levels that limit their capacity to detoxify AscOOH. Higher first week urinary AscOOH levels are associated with an increased incidence of BPD or death.

3.2 Introduction

Recent reports indicate that about 11% of worldwide live births are born preterm with increasing preterm birth rates between 1990 and 2010 [1]. Due to their gastrointestinal immaturity, extremely premature infants depend on parenteral nutrition (PN) during their first days of life. The average time to reach full enteral feeding ranges between 14 and 29 days of life [2, 3].

Oxygen and peroxides contaminants in parenteral nutrition solutions are considered the two main sources of oxidants in the neonatal intensive care unit (NICU). Peroxides contaminating the parenteral nutrition come from the oxidation of polyunsaturated fatty acids, amino acids and vitamin C in the presence of the dissolved oxygen in the PN solution [4, 5]. H_2O_2 constitutes more than 80% of these peroxides [6]. Early exposure to these oxidants has an additive effect on oxidative stress and the development of bronchopulmonary dysplasia (BPD) [7].

At the cellular level, lethal effect on endothelial cells was documented at the concentration of peroxides contaminating the parenteral nutrition with the cells derived from female infants being more resistant to peroxides [8].

In the parenteral nutrition solution, hydrogen peroxide and dehydroascorbate interact to spontaneously generate AscOOH (2,3-diketo-4-hydroxyperoxyl-5,6-dihydroxyhexanoic acid) [4, 9]. Therefore, every individual on parenteral nutrition is infused with this exogenous molecule. Once in the body, AscOOH is detoxified by glutathione peroxidase using glutathione as cofactor [10]. The low glutathione level reported in premature newborn [11] can limit detoxification of AscOOH in this population.

Animal studies suggest that it is the peroxidized metabolite of vitamin C, ascorbylperoxide (AscOOH), rather than hydrogen peroxide that causes hypo-alveolarization and increased apoptosis in lung tissue [12, 13]. Hypo-alveolarization is the main pathologic feature of BPD [14, 15]. This major complication has many short- and long-term consequences on the health and development babies born prematurely [16, 17].

We sought to study how plasma total glutathione, glutathione peroxidase enzyme (GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2) and early urinary levels of AscOOH in preterm infants relate to their later development of BPD or death. We hypothesize that higher urinary levels of AscOOH in those early samples reflect insufficient glutathione antioxidant system and is associated with higher rates of BPD or death.

3.3 Methods

3.3.1 Subjects:

All infants less than 29 weeks gestational age, free of major congenital malformations, admitted to our neonatal intensive care unit in the first 24 hours of life, between August 2010 and July 2011 were targeted **(Figure 18).** The study as well as the informed consent form was approved by the Research Ethics Board of the CHU Sainte-Justine (registration number 2792).

3.3.2 Measurements and definitions:

Urine samples were collected on day 3, 5 and 7 of PN for urinary AscOOH. *Urinary AscOOH* was quantified using an Agilent Technologies LC/MS 1100 mass spectrometer (Santa Clara, CA) using L-2-oxo-thiazolidine 4-carboxilic acid as an internal standard as previously described [9]. Bloods samples were collected for plasma GSH measurement and red

blood cell GPx and GR activity on day 7 of PN. Total plasma glutathione was measured using the enzymatic assay followed by spectrometry reading at the wavelength 412 nm [18]. GR activity was measured according to the method of Becker et al [19]. The activity of GPx was measured using a variant of the method for measuring GR activity as previously described [20].

BPD or death by 36 weeks postmenstrual age is a combined outcome where bronchopulmonary dysplasia is defined as the requirement for additional oxygen at postmenstrual age of 36 weeks [21]. Mortality being a competing outcome for BPD, the outcome of BPD/death should be used rather than BPD alone to identify possible risk factors [22].

3.3.3 Local practices

Our local NICU practices for extremely preterm infants included both: 1) Setting O_2 saturation targets between 85 to 94 % for infants on supplemental O_2; 2) Initiating PN on the first day of life at 80 ml/kg/day and increasing by10-20 ml/kg/day to reach the target of 150 ± 10 ml/kg/day. In addition, amino acids amino acids (Amino Acid Injection 10% w/v, Primene®, Baxter, Toronto, Ontario, Canada) **(Table VI)** was started at 2.5 g/kg/d, then increased 0.5 g/kg/day to achieve 3.5±5 g/kg/day. Multi-12 pediatric (Sandoz, Boucherville, QC, Canada) **(Table VII)** was mixed with the amino acid moiety of PN. Infants less than 750 g received 1.5 ml /day of multivitamins while those infants more than 750 g received 2.5 ml/day. Lipids provided from Intralipid® 20% (Pharmacia Upjohn, Baie d'Urfé, QC, Canada) that contain 200g lipids/L on which 20% Soybean oil, 1.2% Egg Yolk Phospholipids and 2.25% glycerine. Lipids are started on day one of life at 1-2 g/kg/day, then increased by 0.5 g/kg/day to achieve 3±0.5 g/kg/day and are administered separately. PN was not protected from light. Minimal enteral feeding was started at 20 ml/kg/day as soon as the medical

condition of the infants stabilized. If the mother chose to breastfeed her infant, maternal breast milk was given to the baby once available during the first 3 days of life otherwise formula milk was started. Minimal enteral feeding was kept for 4 days and then increased progressively by average of 20 ml/kg/day daily if well tolerated by the infant. According to our nutritional protocol, the majority extremely preterm infants are fed mainly through PN during their first 7 days of life.

3.3.4 Data analysis

Data were summarized as proportions, means with standard error of the mean (SE), or median with 25^{th}-75^{th} percentiles. Statistical analyses were performed using SPSS 22 (IBM software, NY, USA). The comparisons were performed using Chi-square, *t*-student test, Spearman correlation (r), linear regression (adjusted r^2) and repeated measure ANOVA (with Greenhouse-Geisser correction applied wherever the sphericity assumption was not met) as appropriate. $p< 0.05$ was considered significant.

3.4 Results

The consent available group was representative of the whole eligible study population **(Table VIII)**.

Using repeated measure ANOVA, increasing number of days on PN was significantly associated with increasing urinary AscOOH level **(Figure 19)**, after Greenhouse-Geisser correction, F (1.72, 77.33) = 7.37, p = 0.001.

The same repeated measure ANOVA shown that infants in the BPD or death outcome group had higher urinary AscOOH **(Figure 20)**, after Greenhouse-Geisser correction, F (1.74, 72.41) = 3.62, p = 0.037.

Repeated measure ANOVA did not show a statistically significant difference between the male and female infants (p = 0.2) neither significant interaction with duration effect of PN on urinary AscOOH (p = 0.4). Linear regression on the tested 3 time-points demonstrated a weak negative correlation between gestational age and urinary AscOOH, with more mature infants displaying lower urinary AscOOH levels (adjusted r^2: day 3 = 0.12, day 5 = 0.24, day 7 = 0.12; p values were < 0.01.). On day 7 of PN, total plasma glutathione was (mean±SE) 1.23±0.14 µmol/l, GPx activity was 3.98±1.25 and GR activity was 0.36±0.01 nmol/min/mg prot. Using spearman correlation, none of the tested elements of the glutathione antioxidant system significantly correlated with urinary AscOOH on day 7 of PN: total plasma glutathione (r = 0.03, p = 0.83), red blood cell GPx activity (r = 0.03, p = 0.85) and GR activity (r = -0.13, p = 0.4).

3.5 **Discussion**

Results of this study put in evidence the incapacity of extremely preterm infants to detoxify the AscOOH leading to its accumulation in their system and significant increase its urinary excretion overtime during the first week of life. In our cohort, infants with higher urinary AscOOH levels in the first week of life were also more likely to develop BPD or death at 36 weeks postmenstrual age. The suggested toxic effect of this molecule can be explained, at least partly, by deficiency of glutathione system mainly characterized by a low plasma glutathione.

3.5.1 Prematurity, glutathione system and ascorbylperoxide detoxification:

For several species, developmental preparation for postnatal oxidative stress during the final 10% to 15% of gestation includes major increase in antioxidant capacity by 150% to 200% increase in the activity of antioxidant enzymes [23, 24]. Extremely premature infants are lacking this preparation and multiple reports have documented their less developed antioxidant system [25-27]. This can explain their incapacity to detoxify AscOOH contaminating the PN solution; the increasing levels of urinary AscOOH reflecting an overwhelmed antioxidant capacity.

Glutathione is the most abundant non-protein thiol defending against oxidative stress. The glutathione system is responsible of detoxifying peroxides, including AscOOH [10], using reduced glutathione (GSH) by glutathione peroxidase (GPx) resulting in the formation of disulfide glutathione (GSSG) and the alcohol form of the peroxide [28]. In order to maintain its redox potential, the cell actively exports GSSG[29]. To avoid depletion in glutathione, glutathione reductase (GR) recycles GSSG into GSH. In this group of extremely preterm infants, GPx and GR activity on 7 days of PN where similar, even higher, than those reported in adult population (3.98±1.25 versus 2.59 ± 0.07 and 0.36±0.01 nmol/min/mg prot. versus 0.14 ± 0.01 nmol/min/mg prot. respectively) [30]. However, our results demonstrate very low level of total plasma glutathione (including both GSH and GSSG) in these extremely preterm infants. Plasma glutathione was 1.23±0.14 µmol/l (mean±SEM), ranged between 0.16 µmol/l and 4.34 µmol/l with a median of 1.02 µmol/l. In 90% of all infants' plasma glutathione was less than 2 µmol/l. Other works have already found total plasma glutathione levels to be lower in term newborns compared to adults (median 2.2 versus 8.1 µmol/l range: 0.7-9.1 versus 2.1-17.3 µmol/l respectively) [31]. In our study extremely preterm infants have even lower plasma

glutathione level than these low levels previously reported in full term neonates leading to ineffective role in the detoxification of AscOOH in these infants. These results further support the work by Jain et al that described deficiency of glutathione in plasma of preterm infants compared to term infants as well [32]. The importance of the plasma glutathione resides in the fact that cells use glutathione (GSSG or GSH) from plasma as source of amino acids, especially cysteine, for its own *de novo* synthesis of GSH. Plasma GSH is mainly derived from the liver. The liver uses the methionine transformation in cysteine for the synthesis of GSH. Our group has previously shown that peroxides from PN inhibit this transformation, leading to low glutathione in tissues such as liver, erythrocytes [33] and lung [12]. The weak levels of glutathione in plasma, with low variation, could explain that we did not observe a significant role of plasma glutathione in AscOOH detoxification

3.5.2 Ascorbylperoxide and BPD or death:

In guinea pig model, main features of BPD including oxidized redox potential, increased apoptosis and decreased alveolar index followed administration of light exposed PN or AscOOH [12, 13, 34]. Speculating from these works, one can expect extremely preterm infants who cannot detoxify this molecule to be at increased risk of BPD. In our study, this association proved significant as infants with higher urinary AscOOH had a higher incidence of BPD or death. To prevent this outcome, two possible interventions could be proposed. The first intervention would be to decrease the amount of peroxides contaminating PN. For example, shielding PN from light is associated with a 50% reduction in peroxides contaminants [35]. Some randomized controlled trials also have demonstrated a decrease in BPD with shielding PN from light [36, 37], while a recent one did not confirm this finding [38]. Recent

meta-analysis confirmed that adequate shielding of PN decreases mortality by half in extremely preterm infants [39]. The second possible intervention would be to increase the activity of the glutathione antioxidant system. Correcting deficient glutathione levels in guinea pigs (from 0.5±0.2 to 14.2±0.4 μmol/l) by adding glutathione to the PN also resulted in the correction of redox potential, a decrease in apoptosis and a normalising of the alveolar index [10]. Multiple studies have tested the effect of increasing cysteine or N-acetylcysteine in the PN of preterm infants without significant clinical impact according to the Cochrane review [40]. This lack of benefit can be explained by the immaturity of the cellular uptake of cysteine in premature infants as reported for leucocytes from both cord blood and tracheal aspirate [41]. We speculate that supplementation of glutathione to resume within normal plasma glutathione level may decrease BPD or death in extremely preterm infants.

3.5.3 Strengths and limitation:

This study had a representative sample of a tertiary North American NICU population with 10% mortality and 72% of infants with BPD or death for extremely preterm infants less than 29 weeks of gestational age. This study was prospectively conducted. The measuring method of AscOOH is well established and was already validated in previous studies. Serial measurement of urinary AscOOH helped in understanding the evolution of this biologically active contaminant of PN and factors affecting it.

Limitations of this study include its relatively limited sample size. Another limitation was having total plasma glutathione, GPx and GR measured only on day 7 so we were unable assess their evolution overtime. We also could not evaluate the correlation between AscOOH and GPx and total plasma glutathione at earlier point of life (day 3 and day 5). Extremely

preterm babies are at high risk of anemia of prematurity and all efforts are made to limit blood samples. However, a previous study showed a significant drop of glutathione on 2nd day of life compared to the first, with a return back to baseline level on the 7th day, which corresponds with the day we obtained blood samples [31]. In addition, in this study photoprotection of PN was not a standard practice. Unfortunately, all current clinically available methods of photoprotection are partial photoprotection methods and hence confer no additional benefit while consuming valuable resources [42]. To be efficient, the photoprotection must be complete, that means: 1) When PN solutions are prepared in pharmacy all compounder they use should be in amber materiel. 2) If the solutions are prepared manually, the lights in the compounding hood must be safe in regard of the light stimulation of the riboflavin. 3) Multivitamin should be photo-protected at the time of sampling from the vials using amber syringes. This should be done immediately once the vial is first open. 4) Before checking the presence of precipitate in the PN bag using ambient light, the bag must be protected by a material that protects against the light, but sufficiently transparent to see the precipitates. 5) The already compounded PN bags waiting to be delivered by pharmacy to the NICU should be put in opaque bags until they are hung at the bedside. PN bags hung at the bedside should be covered with opaque material with a special attention that the bottom of the bag and the area around the tubing are well covered. 6) The tubing between bag and infant must be photo-protected. Aluminum foil is effective, but it is not a practical solution in the NICU, while being very time consuming. While respecting these conditions was feasible with different degrees of success in well-conducted research projects, it is clear that it is difficult to respect all these conditions in daily clinical practice.

3.6 **Conclusion:**

In extremely preterm infants, we documented the significant increase of urinary AscOOH over the first week on PN, their plasma glutathione deficiency and the significant association between early higher urinary AscOOH and an increased incidence of BPD or death. Our study supports the need for further research aiming to improve the quality of PN by either eliminating or decreasing the peroxide contamination of this invaluable solution or correcting extremely preterm infants' innate glutathione deficiency with supplements.

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3.7 References

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3.8 **Tables**

Table VI. Components of Primene 10% received by the newborns.

	Amino acids (g) in Primene 10%	Amino acids (mg) delivered at a rate of 2.5 g/kg/d	Amino acids delivered at a rate of 3.5 g/kg/d
Lysine	11.00	274	384
Glutamic acid	10.00	249	349
Leucine	10.00	249	349
Arginine	8.40	209	293
Alanine	8.00	200	279
Valine	7.60	190	265
Isoleucine	6.70	167	234
Aspartic acid	6.00	150	209
Phenylalanine	4.20	105	147
Glycine	4.00	100	140
Serine	4.00	100	140
Histidine	3.80	95	133
Threonine	3.7	92	129
Ornithine-HCl	3.18	79	111
Proline	3.00	75	105
Methionine	2.40	60	84

Tryptophan	2.00	50	70
Cysteine	1.89	47	66
Taurine	0.60	15	21
Tyrosine	0.45	11	16

Primene 10% (Amino Acid Injection 10% w/v, indicated for the parenteral nutritional support of preterm infants) was started at 2.5 g/kg/d, then increased 0.5 g/kg/day to achieve 3.5±0.5 g/kg/day.

Table VII. Components of the multivitamin preparation received by the newborns

	In 5 mL* vials	Newborn <750 g	Newborn >750 g
Ascorbic acid	80 mg	24 mg	40 mg
Vitamin A	2300 IU	690 IU	1150 IU
Vitamin D	400IU	120 IU	200 IU
Thiamine-HCl	1.2 mg	0.36 mg	0.6 mg
Riboflavin (as phosphate)	1.4 mg	0.42 mg	0.7 mg
Pyridoxine-HCl	1 mg	0.3 mg	0.5 mg
Niacinamide	17 mg	5.1 mg	8.5 mg
d-Panthenol	5 mg	1.5 mg	2.5 mg
dl-α-tocopherol acetate	7 IU	2.1 IU	3.5 IU
Vitamin K_1	0.2 mg	0.06 mg	0.1 □g
Biotin	20 μg	6 μg	10 μg
Folic Acid	140 μg	42 μg	70 μg
Vitamin B_{12} (cyanocobalamin)	1 μg	0.3 μg	0.5 μg

*Recombined (vial 1 (4 ml) + vial 2 (1ml) = 5 ml) Multi/12 pediatric.

The 5 mL preparation also contains 1.4% polysorbate 80 and 7.5% mannitol. Newborns < 750 g receive 1.5 mL/d whereas those > 750 g receive 2.5 mL/d Multi-12 pediatric.

Table VIII. Baseline clinical characteristics of all eligible infants and consent available infants.

	All Eligible infants (n = 116)	**Consent available infants (n = 51)**
Gestational age, mean ± SEM (weeks)	$26^{3/7} \pm {}^{1/7}$	$26^{4/7} \pm {}^{1/7}$
Birth weight, mean ± SEM (gram)	867 ± 21	846 ± 23
Apgar score at 5 minutes[a]	6 (5 - 7)	6 (5 - 8)
SNAP - II score, mean ± SEM	19 ± 1	19 ± 2
Sex : Female, n (%)	49 (42%)	21 (41%)
IUGR[b], n (%)	24 (21%)	8 (16%)
Vaginal delivery, n (%)	41 (35%)	17 (33%)
Antenatal steroid		
Complete course, n (%)	60 (52%)	26 (51%)
Incomplete course, n (%)	44 (38%)	23 (45%)
Suspected chorioamnionitis, n (%)	16 (14%)	8 (16%)
Maternal preeclampsia, n (%)	22 (19%)	7 (14%)
Maternal diabetes, n (%)	16 (14%)	7 (14%)

[a] presented as median (25^{th} –75^{th} percentiles)

[b] IUGR was defined as infants with birth weight less than 10^{th} percentile for gestational age according to Fenton preterm growth chart.

3.9 **Figures**

Figure 18. Recruitment flow chart.

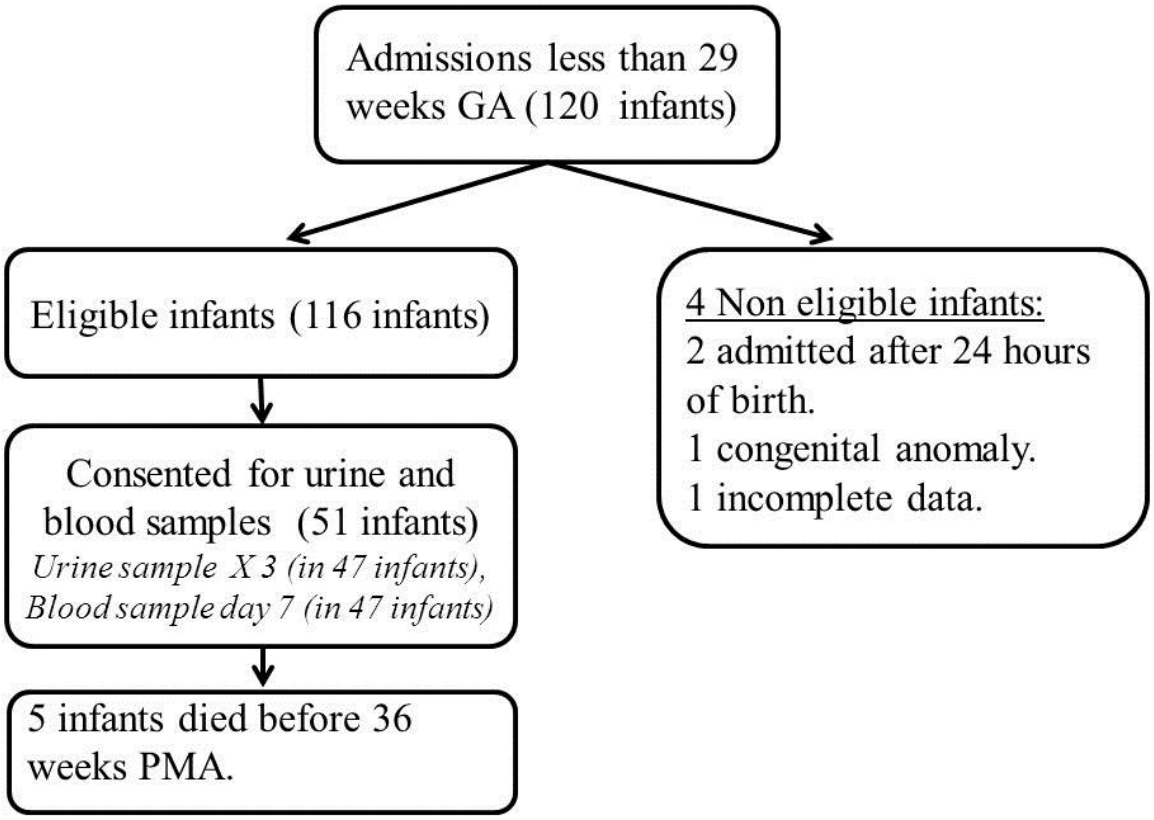
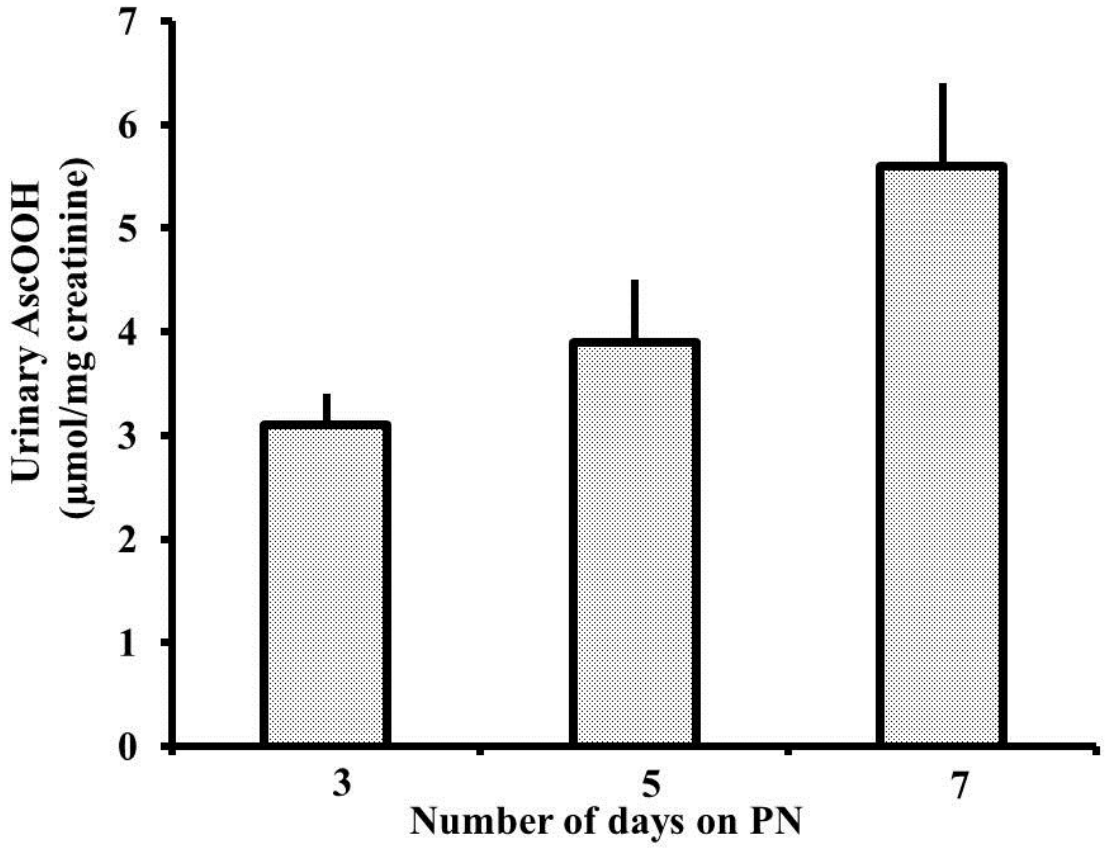
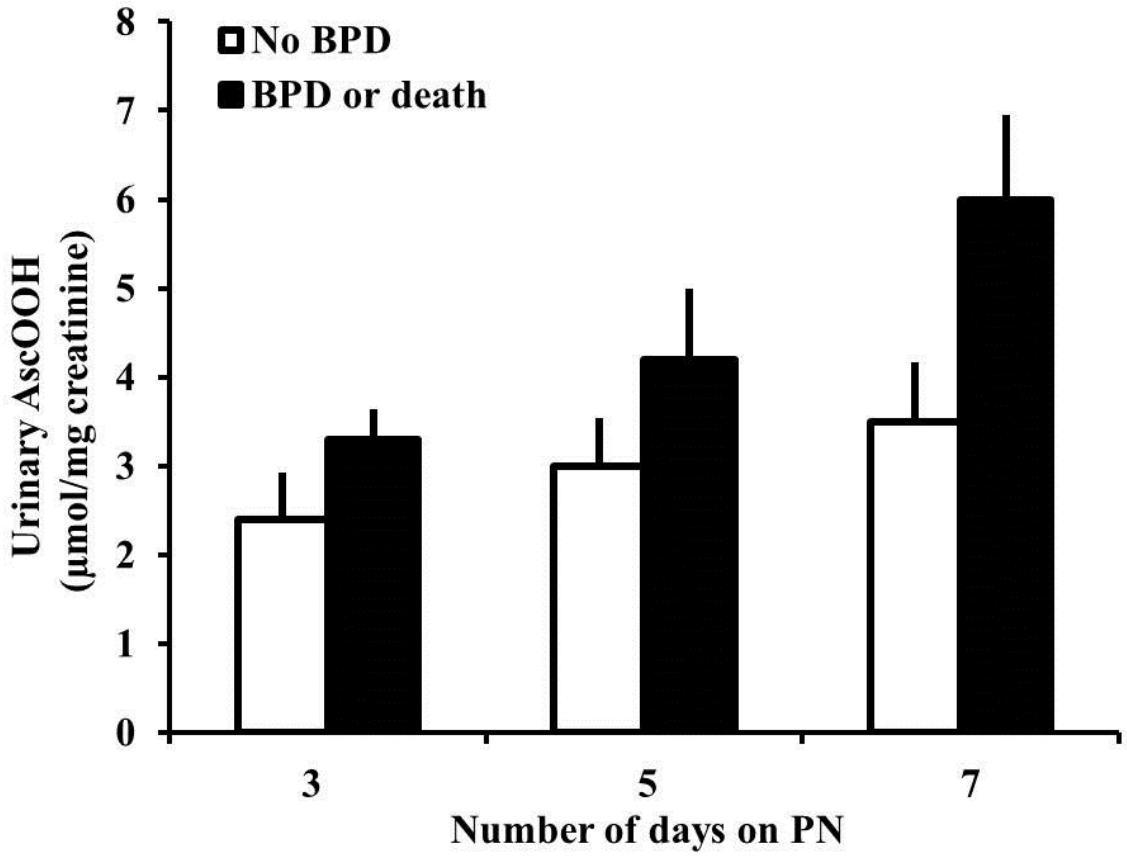


Figure 19. Evolution of urinary ascorbylperoxide (AscOOH) level over the first 7 days of PN.



A significant increase in the whole cohort overtime (p=0.001). AscOOH levels were available on all 3 time-points for 47 infants.

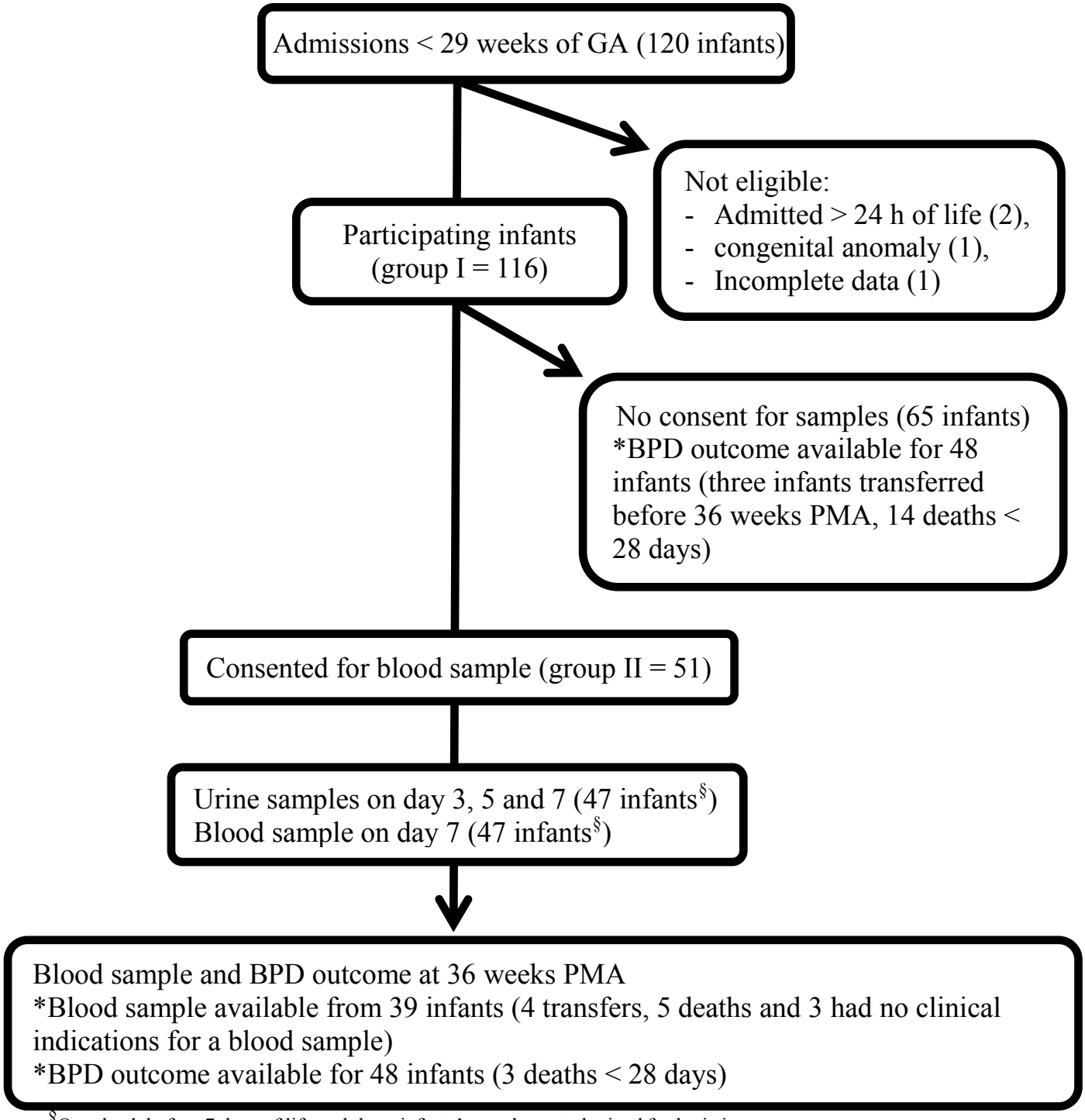
Figure 20. Evolution of urinary ascorbylperoxide (AscOOH) level over the first week of life in relation to BPD or death outcome



The urinary AscOOH was significantly higher in surviving infants with BPD or death (n= 34) versus infants without BPD (n=13) (p=0.037).

4 Chapter 4: Appendices

Figure 21. Combined participants' flow for patients in chapters 2 and 3:



§One death before 7 days of life and three infants' samples not obtained for logistic reasons.

#BPD status could not be determined for infants who died before 28 days of life.

#Infants with consent for biological samples were followed up after their transfer by a phone call to the destination center after 36 weeks PMA to determine their BPD status

Table IX. Basline clinical characteristics of non consent infants versus biological samples consent infants

	infants with no consent (n = 65)	**Consent group (n = 51)**	***P* value**
Gestational age, mean ± SEM (weeks)	$26^{3/7} \pm {}^{1/7}$	$26^{4/7} \pm {}^{1/7}$	0.8
Birth weight, mean ± SEM (gram)	876 ± 32	846 ± 23	0.4
Apgar score at 5 minutes[a]	6 (5 - 7)	6 (5 - 8)	0.7
SNAP - II score, mean ± SEM	20 ± 2	19 ± 2	0.6
Female sex, n (%)	28 (43%)	21 (41%)	0.8
IUGR, n (%)	16 (25%)	8 (16%)	0.2
Vaginal delivery, n (%)	24 (38%)	17 (33%)	0.6
Complete course of antenatal steroids, n (%)	34 (65%)	26 (51%)	0.7
Suspected chorioamnionitis, n (%)	8 (12%)	8 (16%)	0.6
Maternal preeclampsia, n (%)	15 (23%)	7 (14%)	0.2
Maternal diabetes, n (%)	9 (14%)	7 (14%)	0.7

[a] presented as median (25^{th} –75^{th} percentiles)

Table X. Patients' Characteristics and outcomes descriptive statistics:

	Mean	SEM	SD	Median	Minimum	Maximum	IQR
Gestational age (weeks)	$26^{3/7}$	$^{1/7}$	$1^{3/7}$	$26^{5/7}$	$22^{6/7}$	$28^{6/7}$	$2^{2/7}$
Birth weight (g)	863	21	222	820	450	1515	288
SNAP II score	19.5	1.4	15	16	0	69	16
FiO_2 Day 7 of life	31	2	16	24	21	100	14
FiO_2 36 weeks PMA	30	1	13	23	21	80	12
Number of days on PN	27	3	28	20	2	182	22
Plasma GSH on day 7	1.23	0.13	0.92	1.02	0.16	4.34	1.28
TB GSH day 7	6.8	0.4	2.5	6.6	2.7	12.2	4.1
TB GSSG day 7	0.22	0.02	0.17	0.2	0.04	0.94	0.12
TB Redox potential day 7	-194.2	1.5	10.4	-195.3	-212	-165	11
TB GSH 36 weeks PMA	7.5	0.4	2.5	7.1	2.0	12.4	3.1
TB GSSG 36 weeks PMA	0.24	0.02	0.15	0.2	0.07	0.75	0.15
TB Redox 36 weeks PMA	-194.7	1.7	10.3	-194.9	-218	-171	13
Urinary AscOOH on day 3	3.1	0.3	2.1	2.4	0.3	9.9	2.4
Urinary AscOOH on day 5	5.2	1.5	10.1	2.8	0.3	67.9	3.2
Urinary AscOOH on day 7	5.4	0.7	5.2	2.8	0.5	23.6	5.7

* µmol/l, ** nmol/mg protein, # mV, § µmol/mg creatinine

Table XI. Percentiles of patients' characteristics and outcomes:

	5	10	25	50	75	90	95
Gestational age (weeks)	$23^{3/7}$	24	$25^{2/7}$	$26^{5/7}$	$27^{5/7}$	$28^{3/7}$	$28^{6/}7$
Birth weight (g)	560	599	700	820	987	1203	1274
SNAP II score	0	9	9	16	25	45	53
FiO_2 Day 7 of life	21	21	21	24	35	50	59
FiO_2 36 weeks PMA	21	21	21	23	33	46	63
Number of days on PN	7	9	11	20	33	52	84
Plasma GSH on day 7[*]	0.21	0.24	0.49	1.02	1.76	2.46	3.23
TB GSH day 7[**]	2.7	3.2	4.9	6.6	9	10	11.1
TB GSSG day 7[**]	0.07	0.1	0.13	0.2	0.26	0.35	0.7
TB Redox potential day 7[#]	-210	-207	-201	-195	-190	-178	-173
TB GSH 36 weeks PMA[**]	2.3	4.6	5.9	7.1	9	11.5	12
TB GSSG 36 weeks PMA[**]	0.08	0.09	0.14	0.2	0.29	0.45	0.61
TB Redox 36 weeks PMA[#]	-217	-209	-201	-195	-188	-183	-173
Urinary AscOOH on day 3[§]	0.62	1.1	1.6	2.4	4	6.5	7.8
Urinary AscOOH on day 5[§]	0.6	1.1	1.4	2.9	4.6	9.5	19.4

Urinary AscOOH on day 7[§]	0.7	1	1.7	2.8	7.4	14.3	16.77

* µmol/l, ** nmol/mg protein, # mV, § µmol/mg creatinine

Table XII. The effect of FiO_2 on day 7 of life and PN duration on total blood GSH, GSSG and redox potential at 36 weeks PMA:

A- Individual effect of FiO_2 and PN duration:

	FiO_2 < 25% (n = 18)	**FiO_2 ≥ 25% (n = 19)**	**P**	**PN ≤ 14 days (n = 6)**	**PN > 14 days (n = 31)**	**P**
GSH*	7.6 (0.5)	7.4 (0.6)	0.79	7.5 (1.2)	7.5 (0.4)	0.99
GSSG*	0.18 (0.02)	0.29 (0.04)	0.03	0.13 (0.02)	0.26 (0.03)	0.04
Redox potential**	-198 (2)	-191 (2)	0.02	-203 (5)	-193 (2)	0.03

B- Effects of different combinations of FiO2 and PN durations:

	FiO_2 < 25% and PN ≤ 14 d (n = 5)	**FiO_2 < 25% and PN > 14 d (n = 13)**	**FiO_2 ≥ 25% and PN > 14 d (n = 18)**	**P (ANOVA)**
GSH*	8.1 (1.3)	7.5 (0.6)	7.6 (0.6)	0.98
GSSG*	0.14 (0.03)	0.2 (0.02)	0.3 (0.04)	0.045
Redox potential**	-204 (6)	-196 (2)	-190 (2)	0.03

- Mean (S.E.M.); *GSH, GSSG are in nmol/mg of protein, **Redox potential in mV.

- In table B The group of $FiO_2 \geq 25\%$ and PN ≤ 14 had only one patient and it was not included in the analysis.

Table XIII. Risk factors Logistic regression of BPD or death in chapter 2:

	Adjusted OR	95% CI	P Value
Gestational age	3.3	1.13 - 9.9	0.03
SNAP II score	1.14	1.01 - 1.29	0.03
Significant PDA	26.4	1.66 - 419	<0.02
Nosocomial infections	1.8	0.32 - 9.96	0.49
Blood transfusion	0.75	0.47 - 1.19	0.22
FiO_2 on day 7	1.86	1.15 - 3.02	0.02
Duration of PN (days)	1.26	1.07 - 1.49	0.01

- Significant PDA represents only treated PDA (either medical or surgical treatment), nosocomial infection represents the number of episodes of culture proven septicemia, urinary tract infection or meningitis, and blood transfusion represents the number of episodes of blood transfusion.

5 Chapter 5: General discussion

5.1 Summary and novel contributions

While the past few years have witnessed a significant decrease in the mortality of extremely preterm infants, the rates of BPD remain stagnant. BPD in extremely preterm infants is a severe complication that affects a preterm infant's health throughout its life. It also has a significant social and economic impact. Having a good understanding of the contributing factors is vital to finding specific preventive strategies. While BPD is classically related to oxidative stress caused by high FiO_2 exposure, the role of other oxidants is less studied. PN contamination with peroxide is a good example of non-radical oxidative stress (redox stress) and new information about its role in the development of BPD is still emerging. The importance of the effect of PN contamination with peroxides comes from its universality and timing. Indeed, all extremely preterm infants are exposed to PN for days, if not weeks. Also, this exposure occurs very early in their postnatal life when many preterm infants are exposed to O_2 supplementation for respiratory distress and their antioxidant defences are very immature. In this thesis we addressed few questions of importance to understand the link between the redox stress caused by PN contamination with peroxides and the development of BPD.

In chapter 2, we were able to demonstrate that the redox stress caused by PN contamination is not limited in time to the duration of PN as suggested by the work of Chessex et al (146) but rather it is associated with long-term redox stress at 36 weeks PMA for more than 8 weeks following the exposure to PN. This observation joins the findings of Vento et all both in full term and preterm infants that initial delivery room resuscitation can be associated with prolonged oxidative stress for up to 28 days (301, 302). In many situations, the endogenous

antioxidant capacity and the stimulation of antioxidant activities post oxidant exposure are sufficient to neutralize the oxidant and to return the redox potential to its baseline levels in what is defined as acute redox stress. If the cells cannot neutralize the oxidant even with stimulated antioxidant activities, the oxidant level will remain high and the redox level will stabilize at oxidized quasi-stationary level leading to different modifications in cellular homeostasis. This is known as chronic redox stress (242, 277, 303). In the case of extremely preterm infants this incapacity of neutralizing oxidants is expected due to both the immaturity of antioxidant system and the repetitive exposure to multiple oxidants. Another hypothesis is that early redox stress during this window for epigenetic changes can lead to specific epigenetic changes leading to chronic oxidative stress. It was recently demonstrated that early PN exposure is associated with long-term, hypomethylation of DNA in a guinea pig model (304).

It is important to note that in chapter 2 we were able to show the additive impact of FiO_2 and PN contaminated with peroxides on long-term redox stress and the clinical outcome "BPD". This additive relation indicates that multiple interventions are needed to reduce this redox stress and thus the prevention of BPD can only be obtained through a bundle of interventions including the decrease of exposure to different oxidants.

In chapter 3, we were able to demonstrate the inability of extremely preterm infants to detoxify peroxides and specifically measure the PN related peroxide AscOOH. The accumulation of AscOOH and its increase in preterm infants' urine over the first week of life highlights the inability of their body to neutralise this toxic molecule. We were able to demonstrate the effects of AscOOH in our lab guinea pig model (17). As early postnatal levels of AscOOH are significantly higher in infants who will develop BPD or death, compared to

BPD free survival, it may serve as an early biomarker for future BPD risk. Dosing AscOOH early in life may help select infants who will most benefit of the interventions aiming to BPD prevention

In chapter 3, we also explored the issue preterm infants' deficient ability to detoxify peroxides. It is known that non-radical stress caused by peroxides is primarily detoxified by the GSH antioxidant system, requiring GSH, GPx and GR. In this thesis, we found that the lack of substrate (GSH) is the main obstacle.as the activity of the enzymes is comparable to that of adults This finding confirms the results of multiple studies reporting low GSH levels in preterm infants, and demonstrates that even with recent PN amino acids and nutrition regimens, extremely preterm infants still have very low GSH levels (217, 218, 285, 305). This could be due to many factors. First, peroxides contaminating PN inhibit MAT activity preventing the synthesis of cysteine from methionine in guinea pig model. Second, low levels of hepatic cystathionase activity limit cysteine synthesis from methionine. Finally, using modern AA solution, containing more cysteine as Primen©, is inadequate as cellular cysteine uptake activity is decreased in extremely preterm infants; they cannot utilise it for GSH synthesis (18, 306, 307). With these findings, we opened the door for GSH replacement therapy to be tested as a strategy for BPD prevention. We started testing this possibility in our guinea pig model with promising results (19).

5.2 Why choosing urinary AscOOH?

- Several methods were used to measure different urinary peroxides in preterm infants. Many studies in preterm infants used the general peroxide assay, ferrous oxidation of xylenol orange (FOX) assay (1, 4, 278). This method measures both H_2O_2 and lipid

hydroperoxides in the urine. In contrary, our work is measuring only on specific peroxide (AscOOH).

Knowing that H_2O_2 account for approximately 88% of urine peroxide in neonates, one can expect that the FOX results will depend mostly on H_2O_2 concentration. It was demonstrated that H_2O_2 is very variable in the same healthy patient during the same day (308). It is also affected by nutrition; increased after coffee and deceased after green tea consummation (309, 310). H_2O_2 is also markedly increased in ARDS and septicemia (311). This makes H2O2 a very nonspecific measure (312).

On the other hand, while AscOOH is a recently recognized peroxide formed in the PN, animal model data shows that its level is very minimal in animals not on PN (95% confidence value from group without infusion of ascorbylperoxide = 0.80 μmol/mg creatinine) and that the initiation of PN induces a very large change in the urinary AscOOH (14). These criteria suggest that AscOOH may be a useful marker of oxidative stress in preterm infants on PN.

5.3 **Limitations**

While our clinical work discussed in this thesis advanced our knowledge in specific areas, there are limitations. Some of these limitations are inherited to this type of translational research and some are due to ethical or logistic issues. We did our best to minimize the impact of these limitations and to compensate with more ethical or practical strategic alternatives. Here I discuss the main limitations and how we tried to address them:

5.3.1 The non-interventional /analytical /cohort approach

In the clinical works presented in chapter 2 and 3, we used an analytical prospective cohort design knowing that there would be several confounders. For example, high O_2 need on day 7 of life or longer duration of PN could indicative of greater illness severity, which may explain the propensity of these infants to developing BPD.

Oxygen and the use of PN are guided by clinical protocols and it is not acceptable to randomise infants to high O_2 supplement if it is not clinically indicated. Therefore, from the ethical standpoint the most appropriate design for our study was observational/analytical . On the other hand, we used all the appropriate statistical methods to adjust for confounders. For example, we addressed illness severity by using the SNAP II severity score in logistic regression analysis. We also tested for all other potential confounders and integrated all significant ones in our logistic regression analysis.

5.3.2 The limited number of cases

Data emerging from large cohorts tend to be more positively received than those from smaller ones. This is usually true and reasonable for epidemiological associations. However, in works involving translational research, a relatively small cohort with an animal model study as its backbone can represent very powerful evidence.

Another point that should be considered is that all non-interventional analytical clinical studies usually have lower rates of participation as there is no anticipated immediate benefit for the participants undertaking all inconvenience of blood and urine sampling. Despite this, we feel that the number of participants used in our work was reasonable enough to avoid type II statistical error.

5.3.3 The limited number of samples

For the purposes of this work, it may have been interesting to document GSH antioxidant system activity which includes GSH, GPx, GR and correlate these measures with urinary peroxide AscOOH over the first week of life (on day 3, 5, 7 of life and not only on day

7). This would have provided an exhaustive analysis of the main antioxidant system under the condition of non-radical oxidative stress. In addition, having periodic measures of the redox potential over a longitudinal period of time could have helped study the relation between the redox potential and various clinical interventions and complications.

While the above measures would have been helpful, we were limited by our patients' small total blood volume (less than 85 ml for babies less than 1000 g). We sampled as little blood as possible to answer our specific question. We also timed these blood draws concurrently with clinically indicated blood draws to avoid any unnecessary heal sticks or venous punctures with the associated pain and possible complications.

5.4 Strengths

5.4.1 Our research questions

The importance of our research questions derives from the recent increase in the survival of extremely preterm infants with a parallel increase of the total number of surviving infants with BPD. It is estimated that between 10000 and 15000 new cases of BPD develop each year in the United States alone (313). The rising incidence of BPD also produces well documented short-term and long-term health and economic impact on society (37, 39, 40, 102, 106, 121, 130, 133, 134, 314-321). When this work started, there was already established evidence of the negative pulmonary effects of PN peroxide contamination in guinea pig model (10, 13, 296, 297). Studies in the same model demonstrated that PN contamination with peroxides was decreased and the adverse pulmonary effects were reversed with complete photoprotection of the PN (10, 297). Clinically it was confirmed that PN is contaminated with

peroxides and that this contamination could be reduced in the urine of preterm infants when using complete photoprotection (1, 3, 5, 284, 322). In preterm infants the effect of complete photoprotection was favorable on decreasing BPD in limited size randomized controlled trials from different centers (6, 147) and the association between early redox stress and the severity of BPD was confirmed (146). Knowing that the etiology of PBD is a multifactorial it was legitimate to ask the question about the duration of this redox stress and how it interrelate to other strong oxidants to which preterm infants are usually exposed during their initial hospital stay. As our work is translational, we wanted to investigate in human preterm infants the role played by specific peroxides contaminating the PN like AscOOH in the development of the disease through the correlation of the concentration this peroxide early in life and the risk of BPD development later in life. We also wanted to confirm if the lake of the detoxification of peroxides contaminating PN was due to the low GSH as suggested by animal model. The answer to these questions seemed extremely important to us as it can provide new strategies for BPD prevention.

5.4.2 Methodology used in our research

We built our methods on a prospective cohort analytical non-interventional model. Despite the limitations of this model discussed in (4.3.1), it permitted us to evaluate our question about the interaction between different oxidants in real current NICU set-up with current clinical protocols. The prospective nature of the study permitted obtaining detailed phenotyping of all participants and adjustment for all important confounders.

As our questions were precise, we were able to limit our number of samples, and the volume of each sample. We also have chosen to combine all our blood sample with a clinically

indicated blood sample, so we guaranteed to all participant not to have any additional complications or inconvenience cause by an extra blood sample. We used the redox potential of glutathione as an indicator of cellular redox environment instead of the usually used GSH to GSSG ratio that can be misleading. The half-cell reduction potential using Nernst equation provides a better assessment of cellular redox environment as the GSH in the cell ranges from 1 to 10 mM and this relative GSH to GSSG does not account for the total GSH concentration. For example, in a cell having 10 mM GSH a redox potential of -228 mV corresponds to the ratio of GSH to GSSG of 16.6 while if the cell is having 1 mM GSH this ratio should be 166 to have the same redox potential. While using the GSH to GSSG ratio in this example will lead to a conclusion that the 10 mM cell has a significantly more oxidized environment and then it should be in a different biological state, in fact both cells are having similar redox potential suggesting similar biological state (270).

Lastly, we used most appropriate statistical analyses and statistically indicated adjustments to account for different confounders. This was important to counteract the effect of different baseline confounders in a prospective non-randomized cohort.

5.5 Current insights and directions for future research

5.5.1 Putting the puzzle pieces together:

The etiology of bronchopulmonary dysplasia is multifactorial and undoubtedly understanding the role of each factor and its relation to other contributing factors is an essential starting point for the prevention of this devastating disease. These contributing factors are prenatal, perinatal and postnatal as we discussed early in chapter 1 of this thesis.

Postnatal factors are probably the ones we have most control over and the ones that we can actively modify. Here in chapter 2, we describe the important role of the peroxides contaminating PN in inducing a long-lasting redox stress that is having an additive effect with the redox stress resulting from O_2 exposure, the most classically incriminated factor in BPD etiology (323). This redox stress is associated with the adverse pulmonary outcome and here again the effects of both FiO_2 and the longer period of PN exposure are additive (323). In our logistic regression these two oxidants (FiO_2 and peroxides contaminating PN) were the most important modifiable contributing factor to BPD development and its severity. In chapter 3, we were able to demonstrate the strong association of the urinary concentration early in life of a specific PN peroxide (AscOOH) and the later development of BPD (324). We were also able to identify where is the defective point in the antioxidant mechanism involved in the detoxification of peroxides in general including the peroxides contaminating the PN which was the severe GSH deficiency in extremely preterm infants (324). This work provided an important piece of information that suggests specific strategies that will very likely help in BPD prevention.

5.5.2 What is next?

The results provided in this work and in our guinea pig model suggest that oxidative stress as translated to redox stress plays an important role in BPD development. Multiple interventional studies targeted oxidative stress modulation in the neonatal period including trial using less O_2 in the delivery room resuscitation (301), the targeting lower saturation limit (142, 325-327), SOD replacement studies (328, 329), photoprotection of PN from light (6, 147, 330), vitamin A (70) and N acetyl cysteine (331) supplement had variable results. With

the information provided from our work I believe that strategies aiming to BPD prevention should have a comprehensive approach that targets resuming preterm infants' oxidant-antioxidant balance in what I suggest as **Ne**onatal **O**xidant-antioxidant **Balance** research program **(NeO Balance)**. This suggested program needs to have 2 main components: the first includes all interventions aiming to expose preterm infants to **less** oxidative **stress** (**Less stress)** and the second component includes all interventions targeting to **I**mprove how preterm infant can **defend** against oxidants (**I defend)**. Resuming the redox balance in this vulnerable group will permit normalization of many physiological functions, shift cell cycle from apoptosis to proliferation and maturation which we speculate can contribute to BPD prevention. This new approach is depicted in (Figure 21).

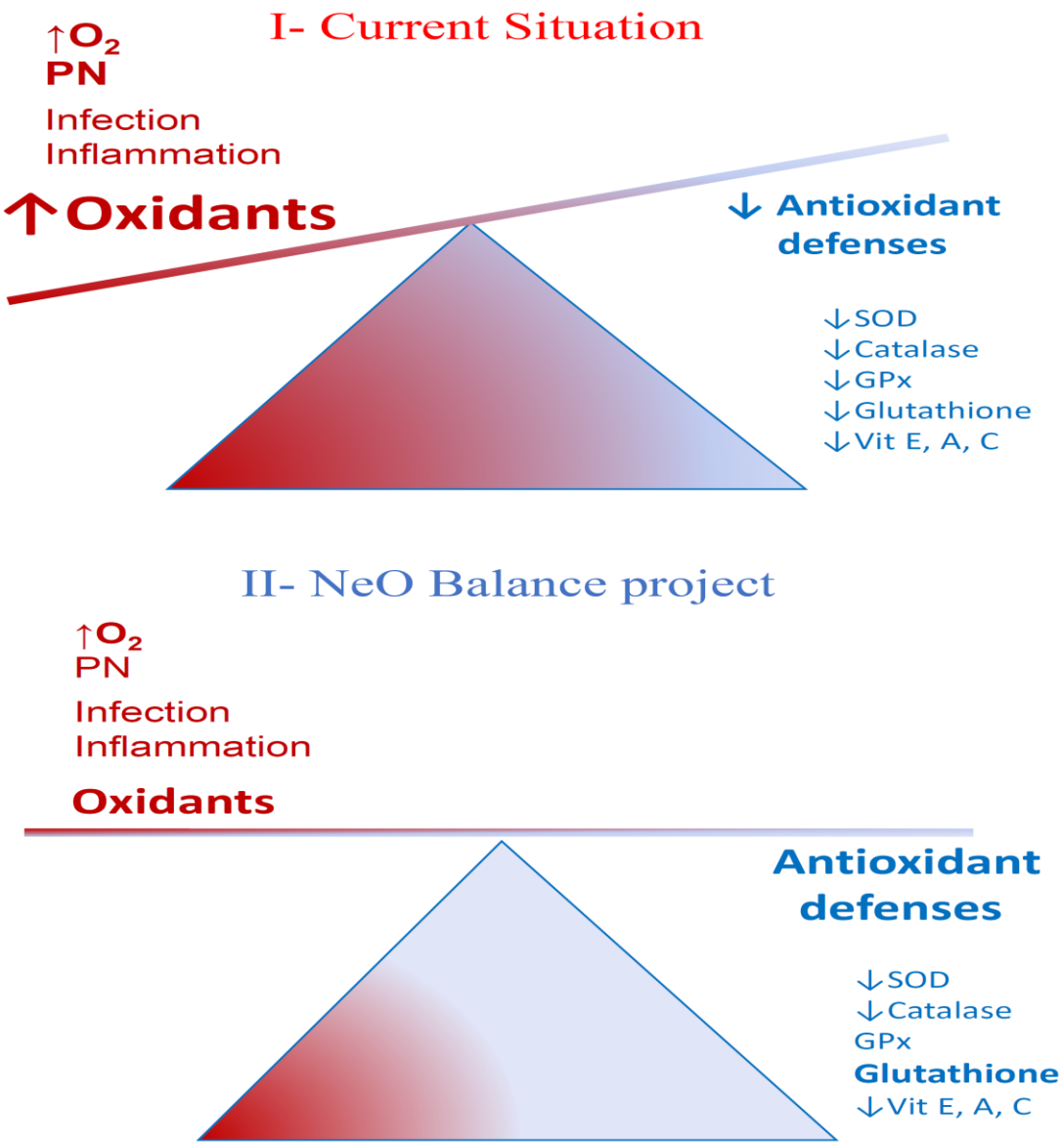


Figure 22. Preterm infants' oxidant-antioxidant status and the Neo Balance project

In the next sections I will present some of our current/future works that are part of this program:

5.5.2.1 *Less stress*: photoprotection and other interventions

We believe that the first step toward the *NeO Balance* objective is to decrease all present interventions that increase the redox stress and to evaluate any new interventions to avoid any one that can negatively impact the redox balance.

Following our work for this thesis the need for a strategy that can decrease PN contamination with peroxides was evident to us. Previous animal studies and single center studies using complete photoprotection since its compounding through its infusion established its beneficial effect on pulmonary outcome (2, 5, 6, 147, 297). It is evident that such method of covering the PN infusion set tubing with photo-opaque material is burdensome and difficult to apply in clinical practice. The multicenter trial that aimed to widely apply this intervention failed to show any benefit and this can be explained by the inherited difficulty to apply the intervention and the center to center variabilities (330). From *in vitro* and human works, exposure of PN to light even for few minutes could cancel any beneficial effect of photoprotection and partial photoprotection was proven to have no benefits (2, 4, 9, 332). We are proposing a project that present a new method of administrating multivitamins in separate photo-protected line in what we called **S**eparating and Shielding **M**ultivit**a**mins to **R**educe **T**oxic **P**eroxides contaminating the **P**arenteral **N**utrition **(SMART-PN)** Study.

We already started *in vitro* part of this study with very encouraging results that demonstrate about 45% decrease in peroxides contaminating PN when using this method. An abstract from this work is accepted for presentation in the Pediatric Academic Societies meeting (PAS meeting) in May 2018. We plan to start a pilot SMART-PN clinical study in the next few months followed by a randomized controlled trial according to the results of the pilot study.

On the other hand, the *less stress* part of the program is extending to include the evaluation of existing or new interventions in the NICU. As SMOFlipids was recently introduced to many NICUs; we sought to evaluate its effects on the redox potential and pulmonary outcome in our guinea pig model. Our results indicated more redox stress and persistence of hypo-alveolarization compared to the regular intralipid solution. In our published work we conclude that further research is needed before routine use of SMOF in the NICU (333). This work represents the first part toward the *NeO Balance* objective with the objective to decrease all present interventions that my increase the redox stress and to evaluate any new interventions to avoid any one that can increase it.

5.5.2.2 *I defend*: improving the antioxidant defenses through GSH replacement

Following the results from our work and the positive results of GSSG supplement to PN solution in guinea pig model, we obtained a fund from the Canadian institute of health (CIHR) research in which I'm co-PI. Our study 'Preventing bronchopulmonary dysplasia in preterm infants by adding glutathione to their parenteral nutrition, a translational study' was funded by the Canadian health institute of health research (CIHR) with a total of 753 000 $ grant for five years period (2016 to 2021) to establish the minimal effective dose of GSSG supplement in PN in guinea pig model and to start a phase I-II clinical study to confirm the minimal dose required to restore the redox balance with no clinical side effects.

Our guinea pig model part of this study is completed with promising results. The addition of glutathione in the parenteral nutrition prevented pulmonary redox stress and maintained alveolar integrity with a specific minimal dose at which this effect starts while higher doses did not show additional benefit. This dose provides an initial dose to study in preterm infants.

These results were accepted to be presented in the next PAS meeting in May 2018. The manuscript of this work is almost finalized for submission. We are presently preparing for the phase I-II clinical study.

5.6 **Conclusions**

Early O_2 supplement and peroxides contaminating PN are associated with prolonged oxidative stress and increased risk of BPD. The effect of these two oxidants was additive. Extremely preterm infants have low glutathione level that limits their capacity to detoxify peroxides contaminating PN including AscOOH. Higher first week urinary AscOOH levels are associated with an increased incidence of BPD or death. A comprehensive approach to resume oxidants-antioxidant balance in extremely preterm infants using interventions like clinically applicable complete photoprotection of multivitamins and GSH replacement therapy represent promising strategies to decrease the risk of BPD development in extremely preterm infants.

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